

**ACTIVIN AND NEUROTROPHIN REGULATION OF
HUMAN FOLLICULAR DEVELOPMENT
AND BOVINE OOCYTE MATURATION**

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ABSTRACT

Primordial follicles are the essential functional units of the ovary and are formed during fetal life in humans. Fetal ovary development is a complex process, dependent on maturation and differentiation of several cell types with differing functions. However, the factors involved in regulation of this growth and differentiation are not yet well understood. Ovarian development and organisation is important for normal reproductive potential. Oocyte competence to support early embryo development is acquired gradually over the course of oogenesis and folliculogenesis and is completed during meiotic maturation. This aim of this thesis was to investigate possible roles for growth factors, namely activin and inhibin, and neurotrophin-4 (NT4) and brain derived neurotrophic factor (BDNF), in fetal ovary development. The effects of BDNF on maturation of the bovine oocyte as well as implications for embryo development after parthenogenetic activation were also investigated.

Activins and inhibins are members of the transforming growth factor β (TGF β) superfamily. A substantial body of evidence has accrued that activins and inhibins are regulatory factors in the adult ovary involved in a wide range of functions. Activin is also expressed in the human gonad at very early stages of development. Expression of mRNA for the activin β A and β B subunits and activin receptors was demonstrated in the human fetal ovary at 14-21 weeks gestation. Quantitative expression of β A mRNA increased two fold across the gestational range examined. Activin subunits and receptors ALK4, ActRIIA and ActRIIB, were localised by immunohistochemistry. The β A

subunit was expressed by oogonia. The β B subunit and activin receptors were expressed by both oogonia and somatic cells. Expression of β A was increased in larger oogonia at later gestations, but was low in oocytes within newly formed primordial follicles. The activin-binding protein follistatin was not detected by immunohistochemistry, nor was the α inhibin subunit. Culture of fetal ovary fragments with activin A increased the number of oogonia present and oogonial proliferation, as detected by BrdU incorporation, indicating a role for activin in the autocrine / paracrine regulation of germ cell proliferation during the period of development leading up to primordial follicle formation.

Neurotrophins are survival and differentiation factors originally identified in the nervous system. Expression and localisation of NT4, BDNF and their common receptors TrkB and p75 was detected in mid-trimester human fetal ovaries at 14 – 19 weeks gestation, indicating a possible role in regulation of proliferation or survival of germ cells or their supportive somatic cells. TrkB signalling appears to be central to the normal formation of primordial follicles that occurs in the ovary in the few days following birth in the rodent. The effect of NT4 and BDNF on oogonial survival and proliferation was investigated using an *in vitro* tissue culture model. Culture of fetal ovary fragments with BDNF, NT4, anti-NT4 or both NT4 and anti-NT4 showed no statistical difference compared to control groups, both in terms of germ cell number and oogonial proliferation. However culture of fetal ovary fragments with BDNF and anti-NT4 showed a decrease in number of germ cells, and reduced proliferation, supporting a

theory of differential ligand signalling via Trk B / p75 receptors in the human fetal ovary prior to primordial follicle formation.

There is also increasing evidence of a role for neurotrophins in adult ovarian development and function, including oocyte maturation. BDNF is present in human follicular fluid, and is secreted by cumulus cells in response to cAMP, suggesting that its production is stimulated by gonadotrophins. RT-PCR and immunohistochemistry demonstrated that bovine cumulus and oocytes express mRNA and protein for BDNF and the p75 receptor. However, mRNA for full length and truncated isoforms of the TrkB receptor are only detected in cumulus, suggesting that oocytes and cumulus differ in their capacity to respond to neurotrophin signalling. In *in vitro* maturation experiments, the proportion of cumulus oocyte complexes maturing to metaphase II was not altered by BDNF using a serum-free maturation system. However, after maturation, the proportion of parthenogenetically activated oocytes forming blastocysts was higher for groups matured with BDNF, an effect that was reversed by maturation in the presence of a BDNF blocking antibody. Similar effects on progression to MII and blastocyst formation were observed using oocytes matured without cumulus. These results suggest neurotrophins play a role in oocyte maturation by promoting oocyte cytoplasmic competence to support embryonic development.

DECLARATION

Except where due acknowledgement is made by reference, the studies undertaken in this thesis were the unaided work of the author. No part of this work has been previously accepted for, or is currently being submitted in candidature for another degree.

Dr Sarah Justine Martins da Silva

PUBLICATIONS

The following publications have arisen from work undertaken in this thesis:

Martins da Silva SJ, Bayne RA, Cambray N, Hartley PS, McNeilly AS and Anderson RA (2004) Expression of activin subunits and receptors in the developing human ovary: activin A promotes germ cell survival and proliferation before primordial follicle formation. *Dev Biol* 266, 334-345.

De Sousa PA, da Silva SJ and Anderson RA (2004) Neurotrophin signaling in oocyte survival and developmental competence: a paradigm for cellular totipotency. *Cloning Stem Cells* 6, 375-385.

Martins da Silva SJ, Gardner JO, Taylor JE, Springbett A, De Sousa PA and Anderson RA (2005) Brain-derived neurotrophic factor promotes bovine oocyte cytoplasmic competence for embryo development. *Reproduction* 129, 423-434.

Publications achieved associated with work not reported in this thesis include:

Bayne RA, Martins da Silva SJ and Anderson RA (2004) Increased expression of the FIGLA transcription factor is associated with primordial follicle formation in the human fetal ovary. *Mol Hum Reprod* 10, 373-381.

Fulton N, Martins da Silva SJ, Bayne RA and Anderson RA (2005) Germ cell proliferation and apoptosis in the developing human ovary. *J Clin Endocrinol Metab* 90, 4664-4670.

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ABBREVIATIONS

| | |
|-----------------|--|
| ABC-HRP | Avidin Biotin Complex conjugated to horseradish peroxidase |
| ActRIIA | Activin type II receptor A |
| ActRIIB | Activin type II receptor B |
| ALK | Activin-like kinase |
| AMH | Anti mullerian hormone |
| ANOVA | Analysis of variance |
| ATF2 | Activating transcription factor 2 |
| ATPase | Adenosine triphosphatase |
| BDNF | Brain derived neurotrophic factor |
| bFGF | Basic fibroblast growth factor |
| 3 β -HSD | 3 β hydroxysteroid dehydrogenase |
| 17 β -HSD | 17 β hydroxysteroid dehydrogenase |
| β ME | β -mercaptoethanol |
| BMP | Bone morphogenetic protein |
| bp | Base pairs |
| BrdU | Bromodeoxyuridine |
| BSA | Bovine serum albumin |
| cAMP | Cyclic adenosine monophosphate |
| CNS | Central nervous system |
| COC | Cumulus-oocyte complexes |
| CRE | cAMP response elements |

| | |
|-------|---|
| CSF | Cytostatic factor |
| DAB | 3,3'-diaminobenzidine tetrahydrochloride |
| DAPI | 4'6-diamidino-2-phenylindole |
| DMAP | 6'-dimethylaminopurine |
| DNA | Deoxyribonucleic acid |
| dNTP | Deoxynucleotide triphosphate |
| DTT | Dithiotreitol |
| EGF | Epidermal growth factor |
| EGTA | Ethylene glycol bis (β aminoethyl ether)-NNN'-N'-tetra acetic acid |
| ER | Oestrogen receptor |
| ERK | Extracellular-regulated kinase |
| FIGLA | Factor in the germline alpha |
| FGF | Fibroblastic growth factor |
| FSH | Follicle stimulating hormone |
| FCS | Fetal calf serum |
| GAPD | Glyceraldehyde-3-phosphate dehydrogenase |
| GDF | Growth and differentiation factor |
| GITC | Guanidine isothiocyanate |
| GnRH | Gonadotrophin releasing hormone |
| GVBD | Germinal vesicle breakdown |
| hCG | Human chorionic gonadotrophin |
| HDL | High density lipoprotein |

| | |
|----------------|--|
| HEPES | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| HIER | Heat induced epitope retrieval |
| HSOF | HEPES-buffered synthetic oviduct fluid |
| ICSI | Intracytoplasmic sperm injection |
| IGF | Insulin-like growth factor |
| IGFBP | Insulin-like growth factor binding protein |
| IMS | Industrial methylated spirits |
| ITS | Insulin, transferrin, sodium selenium |
| IVF | <i>In vitro</i> fertilisation |
| IVM | <i>In vitro</i> maturation |
| JNK | jun N-terminal kinase |
| kb | Kilobases |
| KL | Kit ligand |
| LDL | Low density lipoprotein |
| LH | Luteinising hormone |
| MAPK | Mitogen-activated protein kinase |
| MEK | MAPK/ERK kinase |
| α -MEM | Minimal essential medium alpha |
| MET | Maternal to embryonic transition |
| MPF | Maturation promoting factor |
| NEC | Non-embryo control |
| NF- κ B | Nuclear factor- κ B |

| | |
|---------------|---------------------------------------|
| NGF | Nerve growth factor |
| NGS | Normal goat serum |
| NRS | Normal rabbit serum |
| NSS | Normal swine serum |
| NT3 | Neurotrophin 3 |
| NT4 | Neurotrophin 4 |
| P450arom | P450 aromatase |
| P450c17 | P450 17 alpha-hydroxylase |
| P450scc | P450 side chain cleavage enzyme |
| PAPP-A | Pregnancy associated plasma protein-A |
| PBS | Phosphate buffered saline |
| PCOS | Polycystic ovary syndrome |
| PCR | Polymerase chain reaction |
| PDE | Phosphodiesterase |
| PI3-K | Phosphatidylinositol 3'-kinase |
| PKA | Protein kinase A |
| PKC | Protein kinase C |
| PLC- γ | Phospholipase C- γ |
| PMSG | Pregnant mare serum gonadotrophin |
| RNA | Ribonucleic acid |
| SARA | Smad anchor for activation |
| SEM | Standard error of the mean |

| | |
|-------------|--|
| StAR | Steroidogenic acute regulatory protein |
| TAK 1 | TGF β -activated kinase 1 |
| TBE | Tris, boric acid, EDTA |
| TBS | Tris-buffered saline |
| TCM 199 | Tissue culture medium 199 |
| TGF β | Transforming growth factor β |
| TNF | Tumour necrosis factor |
| TrkA | Tyrosine kinase receptor A |
| TrkB | Tyrosine kinase receptor B |
| trTrkB | Truncated tyrosine kinase receptor B |
| TrkC | Tyrosine kinase receptor C |
| UV | Ultraviolet |
| VEGF | Vascular endothelial growth factor |

CHAPTER 1

INTRODUCTION

1.1 HUMAN OVARY DEVELOPMENT - INTRODUCTION

Germ cells are not essential for survival of an individual, but are fundamental to the generation of offspring. As such, they hold the key to life, both in terms of survival of a species, as well as totipotency. Oocytes also contribute virtually all cytoplasm to the early embryo. The mechanisms involved in germ cell formation, migration and ovarian development represent the foundations for adult reproductive function, and whilst research has helped define developmental events and pathways in fetal ovarian development and folliculogenesis, much still remains unknown or incompletely understood. In an era of declining fertility, with increasing demand for Assisted Reproductive Techniques, it is vital that these normal mechanisms are better understood. Understanding of developmental and maturational events, and defining the growth factors essential for normal pathways will hopefully enable an increase in treatment options for the subfertile, as well as offering new therapeutic techniques to treat pathological conditions, such as premature ovarian failure.

The following literature review will initially cover the development of the fetal ovary and then review folliculogenesis, including oocyte maturation. Lastly, two families of growth factors that are likely to be involved at various stages of ovarian development will be considered.

1.2.1 HUMAN GONADAL DEVELOPMENT – INDIFFERENT STAGE

Human gonadal development begins in the 4th embryonic week in parallel with the formation of the ventral body wall. Undifferentiated primordial germ cells arise from endoderm lining the yolk sac. Bone morphogenetic protein 4 (BMP4), BMP8b and components of the Smad signalling pathways produced by extraembryonic ectoderm (Ying *et al.*, 2000; Tres *et al.*, 2004; Okamura *et al.*, 2005), in association with endoderm-derived BMP2 (Coucouvani and Martin, 1999; Ying and Zhao, 2001) are involved in the specification of primordial germ cells. Studies with double heterozygote mutant mice have shown BMP2 and BMP4 to have an additive effect on primordial germ cell generation, whereas the BMP2 and BMP8b do not (Ying *et al.*, 2001; Ying and Zhao, 2001). Consequently, two signalling pathways have been suggested to be involved in primordial germ cell development; one for BMP2 and BMP4 and a separate pathway for BMP8b (Ying *et al.*, 2001; Ying and Zhao, 2001).

Primordial germ cells migrate by amoeboid movement along the dorsal mesentery of the hindgut to the genital ridges, which lie on the medial aspect of the mesonephric ridge (Witschi, 1948; Chiquoine, 1954). Factors involved in primordial germ cell migration remain poorly defined although kit ligand (KL, also known as stem cell factor) and its receptor c-kit are required for appropriate migration to occur (Manova *et al.*, 1990; Godin *et al.*, 1991; Loveland and Schlatt, 1997). Adhesion molecules, such as cadherins and integrins, are also thought to play a role in colonisation of the mesonephric ridge and in establishing the architecture of the gonad (De Felici *et al.*, 2005). Failure of

primordial germ cells to develop or populate the genital ridges results in total failure of the gonads to develop.

Over the next two weeks the primordial germ cells mitose repeatedly, forming a population of precursor gametes (Gosden, 1995). Germ cell survival at these early developmental stages is dependent on locally derived growth factors, including BMP4 (Fujiwara *et al.*, 2001a), and kit ligand (Manova *et al.*, 1990; Godin *et al.*, 1991). At this point male and female gonads are histologically indistinct, hence are known as indifferent gonads. In humans, the germ cells are postulated to stimulate the cells of the coelomic epithelium lining the genital ridge to differentiate into a primitive germinal epithelium, hence germ cells become embedded and surrounded by somatic cells (Byskov, 1986).

1.2.2 HUMAN GONADAL DIFFERENTIATION

The Y chromosome is much smaller than the X, and has little deoxyribonucleic acid (DNA) available for ribonucleic acid (RNA) synthesis. Many genes that control testes development are, by necessity, located on other chromosomes. However, the Y chromosome contains a specific single-copy gene, SRY, located in the sex-determining region. The presence or absence of this gene has a direct effect on gonadal differentiation (Koopman *et al.*, 1991). Absence or damage to the sequence results in ovarian development and a phenotypic female (Hawkins *et al.*, 1992). SRY initiates a cascade of activation of genes “downstream” from the Y chromosome and results in differentiation of Sertoli cells, a key cell type in the testis that is essential to subsequent

development. Sertoli cells polarise, aggregate around germ cells and reorganise the gonad into two compartments: the tubular testis cords, composed of Sertoli cells and germ cells, and the interstitial space between the cords (Page *et al.*, 1987). Peritubular myoid cells surround Sertoli cells and co-operate to deposit the basal lamina at the periphery of tubule structures (Skinner *et al.*, 1985; Tung and Fritz, 1987). Other interstitial cell types include steroid-secreting Leydig cells, fibroblasts and endothelial cells that will establish coelomic vasculature. As soon as the testis begins to be differentiated, it starts to synthesise two substances, testosterone and antimullerian hormone (AMH). Testosterone is synthesised by Leydig cells and supports differentiation and growth of Wolffian duct structures (Boehmer *et al.*, 1999). AMH is a glycoprotein produced by the fetal Sertoli cells and causes cranial to caudal regression of Mullerian ducts (Josso, 1972). In the absence of testicular hormones, differentiation continues in a feminine direction (Jost, 1965). Ovary and testes become histologically distinct by the 8th embryonic week.

1.2.3 HUMAN FETAL OVARY DEVELOPMENT AND ORGANISATION

Ovarian differentiation occurs approximately two weeks later than testicular development in humans. The germ cells are termed oogonia and continue to divide mitotically until they enter meiosis. Cytoplasmic cleavage may be incomplete and result in daughter cells remaining linked by cytoplasmic bridges (Gondos *et al.*, 1971), which allows transfer of different substances including growth factors and hormones (Gondos *et al.*, 1973), gene products (Beatty, 1970) or even organelles (Gosden, 1995). These syncytia of germ cells are also termed ‘nests’, and often divide synchronously until

associations begin to break down at meiosis. Binucleate oogonia may form when cleavage is incomplete and can potentially give rise to polyploid embryos. Concurrent with oogonial proliferation, subepithelial mesenchyme invades the gonad, breaking up the germ cell nests, and forming ovarian stroma.

Three major events take place during the early stages of ovarian differentiation: meiosis is initiated, oocytes are enclosed in follicles and steroid-producing cells differentiate once follicles are formed (theca cells) (Byskov, 1986). Once oogonia enter meiosis, they are termed oocytes and no longer possess stem cell potential. In the human ovary, entry into meiosis occurs over a wide timescale. It is detectable as early as 11 weeks gestation (Gondos *et al.*, 1986; Bendtsen *et al.*, 2006), and is maximal around 20 weeks (Baker, 1963). DNA is duplicated prior to meiosis and subsequently undergoes genetic recombination at sites of chiasmata. Meiosis is arrested at this stage (diplotene) of prophase I, and will not proceed until oocytes have grown to full size in a follicle that has been stimulated by gonadotrophins (Hilscher, 1991).

The human fetal ovary contains its maximal number of germ cells (approximately 3.5 million per ovary) by mid-gestation. This reflects increase in germ cell number by mitotic proliferation, which vastly exceeds loss by apoptosis up until this time. Beyond this point the balance is reversed and there is accelerated loss of large numbers of germ cells resulting in approximately one third of the maximal number remaining by term (Baker, 1963) (Figure 1.1).

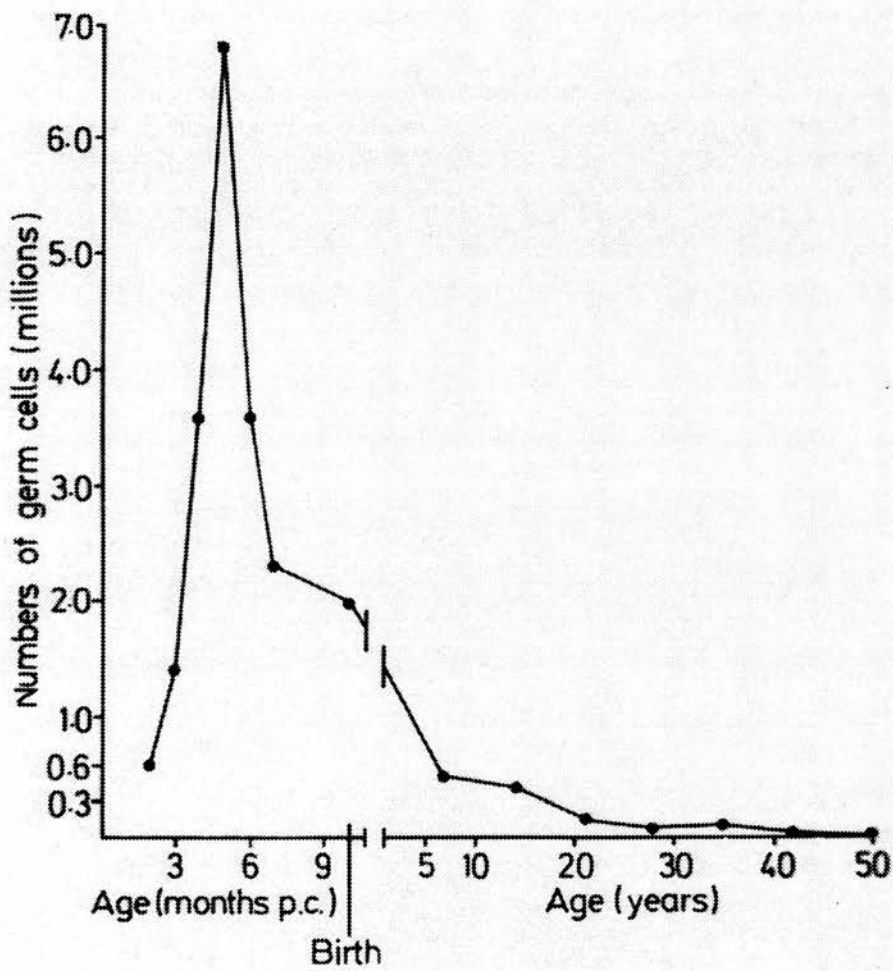


Figure 1.1 Graph showing numbers of germ cells in human ovaries during fetal development and across adult reproductive life (Baker, 1963).

A follicle is formed when somatic cells, the pre-granulosa cells, surround an oocyte and an intact basal lamina encloses this unit (Peters *et al.*, 1978). Follicular formation occurs from mid-gestation onwards and is believed to be crucial to oocyte survival; if an oocyte is not enclosed in a follicle, it degenerates (Faddy and Gosden, 1995). The interaction not only determines oocyte survival by protecting against apoptosis (Fulton *et al.*, 2005) but also enables bi-directional communication between oocyte and somatic cells. By virtue of the basal lamina, granulosa and oocyte exist within a microenvironment without direct contact with other cells. Factors triggering follicular development and organisation are largely unknown, but germ cells are crucial to formation and maintenance of follicles in the ovary; in their absence, follicles degenerate into cord-like structures (Merchant-Larios and Centeno, 1981; McLaren, 1999).

Factor in the germline alpha (FIGLA) is a basic helix-loop-helix transcription factor that binds zona pellucida promoters (Liang *et al.*, 1997). *Figla* is expressed in the female gonad and appears exclusively confined to oogonia / oocytes (Soyal *et al.*, 2000; Bayne *et al.*, 2004). Although *Figla* is also expressed at low levels in testis, only female *Figla* knockout mice are infertile. Deficiency of *Figla* does not affect germ cell migration or proliferation, and embryonic gonads appear normal. However, oocytes rapidly disappear after birth and primordial follicles do not form (Soyal *et al.*, 2000). *Figla* deletion does not affect transcription of other genes preferentially expressed in the oocyte, including growth and differentiation factor 9 (GDF9), BMP 15, kit receptor, connexin 43 and fibroblast growth factor 8 (FGF8), suggesting it may regulate other downstream target genes critical to follicle formation.

Low expression of aromatase enzyme mRNA has been observed in first trimester fetal ovaries (Pakarinen *et al.*, 1990) and the fetal ovary is capable of synthesizing estradiol as early as the 8th week of fetal age (George and Wilson, 1978), although lacks other steroidogenic enzymes necessary for *de novo* synthesis of steroid precursors (Payne and Jaffe, 1974). Whether this secretion of estradiol plays a physiologic role in the human sex differentiation is not known, although there is some emerging evidence that it is required for primordial follicle formation (Billiar *et al.*, 2003; Britt *et al.*, 2004). Thecal cells are derived from mesenchymal stroma, and are not present until follicular growth beyond the primordial stage (Gosden, 1995). Precursor thecal cells are recruited during the transition to primary follicle, but are gonadotrophin and steroid independent and non-steroidogenic at this stage (Braw-Tal and Roth, 2005).

1.3 FOLLICULOGENESIS

Folliculogenesis requires cell proliferation and cytodifferentiation in order to attain successively higher levels of cellular organisation. It includes four major developmental events: 1) primordial follicle recruitment, 2) preantral follicle development, 3) antral follicle development and 4) follicular selection or atresia, and can be divided into two phases: 1) the preantral or gonadotrophin-independent phase, which is characterised by oocyte growth and controlled by locally produced growth factors through autocrine and paracrine mechanisms, and 2) the antral or gonadotrophin-dependent phase, which is characterised by the massive increase in the size of the follicle itself. This phase is regulated by follicle stimulating hormone (FSH) and luteinising hormone (LH), as well

as by growth factors that stimulate cell proliferation and modulate gonadotropin action (Erickson and Shimasaki, 2000).

1.4.1 PRIMORDIAL FOLLICLE RECRUITMENT

The human ovary contains about 1 million oocytes at birth. During childhood the ovary is not a quiescent organ, but contains growing follicles at virtually all stages of development. Lack of appropriate gonadotrophin support results in atresia, thus oocytes continue to decline in number, and only about 100,000 will remain by puberty (Baker, 1963). These oocytes are commonly believed to be finite and will continue to decrease in number until the end of a female's reproductive life, however a recent controversial publication proposed the concept of germ-line stem cells as a renewable source of female germ cells, derived from bone marrow and delivered to the ovaries via the blood stream (Johnson *et al.*, 2005).

Primordial follicles are the fundamental reproductive units of the ovary and give rise to all dominant follicles. There is continuous recruitment from the pool of primordial follicles. As many as 20 follicles begin to mature in each wave of recruitment (Gosden, 1995), beginning with the formation of primary follicles, but typically only one will eventually ovulate. The mechanisms for selection and activation of primordial follicles, and their entry into the cohort of developing follicles, are not completely understood but morphometric studies suggest that these follicles initiate growth based upon the order in which they are formed (Hirshfield, 1991).

Early follicular growth requires kit ligand, produced by the granulosa cells, and the presence of c-kit receptor on oocytes (Elvin and Matzuk, 1998; Parrott and Skinner, 1999). Basic fibroblast growth factor (bFGF) is localised to oocytes of primordial and primary follicles (van Wezel *et al.*, 1995; Nilsson *et al.*, 2001) and is also potent at inducing primordial follicle development (Nilsson *et al.*, 2001). BMP4 has been shown to promote the primordial to primary follicle transition, but also appears to be essential for oocyte survival. When a neutralising antibody to BMP4 was used in organ culture, all oocytes were lost within 14 days (Nilsson and Skinner, 2003). Other members of the TGF β superfamily have also been demonstrated to play a role in the control of onward follicular development, including oocyte-specific factors, GDF9 (Dong *et al.*, 1996; Carabatsos *et al.*, 1998) and BMP15 (also known as GDF9B), (Davis *et al.*, 1991; Braw-Tal *et al.*, 1993), as well as granulosa-derived BMP7 (Lee *et al.*, 2001b; Lee *et al.*, 2004). Activin A, its receptors and the binding protein follistatin are expressed at all follicular stages, indicating a regulatory role of activin A across the entirety of follicular development (Silva *et al.*, 2004). Another TGF β superfamily member, AMH, has been found to inhibit, but not completely block, recruitment of primordial follicles (Durlinger *et al.*, 2002; Visser and Themmen, 2005). In rodents, kit ligand can also promote oocyte growth (Elvin and Matzuk, 1998).

1.4.2 PREANTRAL FOLLICLE DEVELOPMENT

Most oocyte growth is achieved during the gonadotrophin-independent preantral follicular stage. This requires complex cytoplasmic organisation and is dependent on the

production of new gene products and organelles as well as the redistribution and modification of existing ones (Picton *et al.*, 1998). A primary follicle is defined by the presence of cuboidal granulosa cells, which are arranged in a single layer around the oocyte. When more than one layer of granulosa cells surrounds the oocyte, the follicle is described as secondary, or preantral. During this phase of oocyte growth, a glycoprotein membrane is secreted, the zona pellucida, which forms a protective coat around the oocyte. Other events occurring in the preantral follicle during this stage of development include FSH receptor expression in granulosa cells and recruitment of stromal cells to form theca interna and externa. Complex autocrine and paracrine interactions exist between oocytes and their surrounding granulosa cells to ensure growth and differentiation is co-ordinated (Eppig, 1991; Eppig, 2001; Matzuk *et al.*, 2002). Granulosa cells are also coupled with each other, and gap junctions are formed between granulosa and oocyte via processes passing through the developing zona pellucida (Anderson and Albertini, 1976).

TGF β superfamily members expressed by follicular cells and implicated in this phase of development include TGF β , activin (Thomas *et al.*, 2003), GDF9, BMP15 and several other BMPs (Shimasaki *et al.*, 1999; Elvin *et al.*, 2000; Knight and Glister, 2003). BMP6 is expressed in oocytes and granulosa cells (Erickson and Shimasaki, 2003; Souza *et al.*, 2003; Glister *et al.*, 2004) but is derived entirely from oocytes early in follicular development (McNatty *et al.*, 2005). It enhances proliferation of granulosa cells, a process required for follicular growth, and prevents premature luteinisation, a

terminal differentiation event that normally occurs during the periovulatory period. This theory is supported by findings that BMP6 is present in granulosa cells of preantral and antral follicles but not detected in the dominant follicle in rats (Erickson and Shimasaki, 2003). Numerous other growth factors have been identified to orchestrate preantral follicular growth and development, including nerve growth factor (NGF), a member of the neurotrophin family (Dissen *et al.*, 2001), but will not be considered in further detail here.

1.4.3 ANTRAL FOLLICLE DEVELOPMENT

Antral (or tertiary) follicular growth and development can be arbitrarily divided into four stages based on size. In humans, each dominant follicle has a destiny to complete the transition from small (1-6 mm), to medium (7-11 mm), to large (12-17 mm) to preovulatory (18-23 mm) follicle. Atretic follicles usually fail to develop beyond the small to the medium stage (1-10 mm). It is the accumulation of follicular fluid, as well as cell proliferation, which results in the massive growth of antral follicles. The initial signals for the development of a follicular antrum are not well understood, but it is characterised by the appearance of a fluid filled cavity at one pole of the follicle. This process is termed cavitation and two proteins expressed by the follicle itself are essential for it to occur, namely granulosa-derived kit ligand (Driancourt *et al.*, 2000) and oocyte connexin 37 (Simon *et al.*, 1997). If either of these proteins is absent, then no antral follicles develop and the female is infertile.

Follicle development beyond the preantral stage is dependent upon gonadotrophins (Erickson and Danforth, 1995; Kumar *et al.*, 1997) and the final growth of the preovulatory follicle is critically dependent upon FSH (Zelevnik *et al.*, 1985). FSH receptors are also vital to normal progression of folliculogenesis. Generation of knockout mice where all forms of FSH receptor expression are eliminated results in a genetic model known as the FORKO mouse. Mutants are sterile and do not ovulate (Danilovich *et al.*, 2000). The FORKO ovary displays only primordial and pre-antral follicles, with hyperplasia of interstitial tissue in the middle of the ovary (Danilovich *et al.*, 2000). Follicular growth is also dependent on insulin-like growth factor 1 (IGF-1) and its receptor (Zhou *et al.*, 1997; Monget and Bondy, 2000), oestradiol synthesis (Fisher *et al.*, 1998) and the presence of both oestrogen receptor (ER) subtypes, ER α and ER β , (Couse *et al.*, 1999a; Couse *et al.*, 1999b; Couse and Korach, 1999; Dupont *et al.*, 2000) as well as the LH receptor (Lei *et al.*, 2001; Zhang *et al.*, 2001).

FSH binds to its receptor with high affinity. The binding event initiates a conformational change in the receptor that activates G-proteins and the subsequent cascade of events results in generation of cyclic adenosine monophosphate (cAMP). Regulatory subunits of protein kinase A (PKA) bind to cAMP, causing the complex to dissociate and release two free catalytic subunits. The catalytic subunits phosphorylate serine and threonine residues of CREB and CREM proteins, which then bind to upstream DNA regulatory elements known as cAMP response elements (CRE) that act to regulate gene activity.

This FSH control of gene activity in the granulosa cells is the basis of all processes of follicle growth and development, including dominance to the preovulatory stage.

It is apparent that co-ordinated differentiation of the oocyte with its surrounding granulosa cells, and the maintenance of adequate communication between these two cell types, is critical to ensure correct oocyte meiotic maturation (Klinger and De Felici, 2002). Before antrum formation, oocytes are unable to progress beyond the diplotene stage of meiosis I and are described as meiotically incompetent (Sorensen and Wassarman, 1976). This state is attributable to an insufficiency in the regulatory molecules required to drive meiotic progression (Handel, 1998). However, most oocytes in antral follicles, by contrast, are meiotically competent and will resume meiosis spontaneously if removed from the follicle and cultured in supportive medium (Handel, 1998). Meiotic competence is associated with the accumulation of cell cycle regulatory factors (Chesnel and Eppig, 1995) as well as reorganisation of chromatin and microtubule configurations (Wickramasinghe *et al.*, 1991). It is important to note however, that oocytes competent to resume meiosis are not necessarily competent to undergo complete nuclear maturation or progress to metaphase II (Handel, 1998). Growth factors such as GDF9, BMP6 and BMP15, are produced by the oocyte to ensure on-going co-ordination in follicular development. They act directly in granulosa cells to inhibit cytodifferentiation, thus maintaining cell proliferation and follicular growth (Otsuka *et al.*, 2000; Erickson and Shimasaki, 2003; Moore and Shimasaki, 2005).

Around the time of antrum formation, thecal cells begin to express their differentiated state. This event involves the expression of a battery of genes, including LH receptors, insulin receptors, lipoprotein receptors for high density lipoprotein (HDL) and low density lipoprotein (LDL), steroidogenic acute regulatory (StAR) protein, P450 side chain cleavage (P450scc), 3 β -hydroxysteroid dehydrogenase (3 β -HSD), and P450 17 α -hydroxylase (P450c17). Expression of these genes enables thecal cells to produce androstenedione, the androgen substrate required for oestrogen biosynthesis. LH is the most important effector of thecal cytodifferentiation (Erickson *et al.*, 1985), but insulin and lipoproteins can act in synergy with LH to amplify this process. A variety of other regulatory ligands and growth factors have been identified that modulate mammalian theca androgen production, including insulin and IGF-I (Magoffin and Erickson, 1988; Li *et al.*, 2001; Mendez *et al.*, 2005), lipoprotein (Dyer and Curtiss, 1988; Magoffin and Erickson, 1988; Zerbinatti *et al.*, 2001), and various members of the TGF β superfamily: activin, inhibin, BMP4, 6 and 7 and GDF9 (Hillier *et al.*, 1991a; Hillier *et al.*, 1991b; Knight and Glister, 2003; Glister *et al.*, 2005). Except for insulin, the significance of these regulatory molecules is unclear. Insulin receptors with protein tyrosine kinase activity are expressed in human theca cells and their signal transduction pathways have been demonstrated to stimulate androstenedione production. Insulin can increase androstenedione production by itself, as well as synergize with LH to further increase androgen biosynthesis. Observations in rodents indicate that LDL and HDL also stimulate steroidogenesis by human thecal cells (Dyer and Curtiss, 1988; Zerbinatti and Dyer, 1999), and they too can cooperate with LH to cause further increases in

androgen production. Whether increased androgen production by LDL and/or HDL has any physiological meaning is not clear. Nonetheless, it is noteworthy that HDL is the most potent stimulator of theca androgen production known so far. Finally, activin and inhibin can inhibit and stimulate, respectively, androgen production by human thecal cells *in vitro* (Hillier *et al.*, 1991a; Hillier *et al.*, 1991b). Recent studies have found that GDF9 and BMP4 interact with cultured human theca cells to inhibit androgen biosynthesis (Hickey *et al.*, 2005).

The enlarging follicular antrum eventually divides the population of granulosa cells into two main groups: cumulus cells, which are associated with the oocyte, and mural granulosa cells, which line the follicular wall. With advancing follicular development these populations can be distinguished in molecular as well as morphological terms. Cumulus cells express few, if any LH receptors, whereas mural granulosa cells express these receptors, and a gradient of expression is seen with highest expression observed in cells closest to the basal lamina (Amsterdam *et al.*, 1975). Aromatase is expressed in mural granulosa of antral and periovulatory follicles, but is absent from cumulus (Inkster and Brodie, 1991).

1.4.4 FOLLICLE SELECTION AND ATRESIA

Establishing a dominant ovarian follicle capable of ovulating a fertilisable oocyte is undisputed as a fundamental process in female fertility, although the precise mechanism of follicular selection and dominance remains to be fully elucidated. FSH plays an obligatory role in the mechanisms of selection and dominant follicle development and

the overall process is governed by pituitary gonadotrophins, although their effects are almost certainly mediated in conjunction with locally synthesised growth factors. LH is not essential for selection, but is important in regulating dominant follicle formation through its capacity to stimulate the expression of the aromatase substrate, androstendione.

Dominant follicle selection initially appears to depend on differential FSH sensitivity amongst the growing cohort of antral follicles. Activin may contribute to this selection process by affecting the sensitivity of follicles to FSH (Ginther *et al.*, 2002). Conversely, AMH has been shown to decrease the sensitivity of follicles for FSH-dependent selection for dominance (Visser and Themmen, 2005). FSH induces expression of pregnancy-associated plasma protein-A (PAPP-A), which may be a very early marker of dominant follicle selection (Hourvitz *et al.*, 2000). PAPP-A is a protease and decreases the availability of insulin-like growth factor binding globulin-4 and -5 (IGFBP-4 and -5) (Lawrence *et al.*, 1999). Its presence results in an increase in intrafollicular IGF, a growth factor that synergises with FSH to increase oestradiol production (Fortune *et al.*, 2004).

FSH actions are thought to be well defined but recent studies have identified a novel cAMP signalling pathway, where FSH stimulates phosphorylation of kinases that are downstream targets of the IGF-1 / phosphatidylinositol 3'-kinase (PI3 kinase) / PDK1 pathway (Gonzalez-Robayna *et al.*, 2000; Richards, 2001). This impacts differentiation

events by activation of transcription factors and also confers the potential of FSH to maintain cell survival pathways (Brunet *et al.*, 2001).

As the dominant follicle grows, FSH causes the granulosa cells to acquire the potential to produce large amounts of oestradiol. Induction of expression of P450 aromatase (P450arom; the CYP19 gene) in granulosa cells is causal to the acquisition of the oestrogen potential of the follicle (Findlay *et al.*, 2001; Toda *et al.*, 2001) and enables oestradiol synthesis utilising androstenedione supplied by thecal cells. Within the follicle, oestrogen acts in an autocrine and paracrine manner to enhance FSH action on granulosa cells. The resultant augmented aromatase P450 expression and oestradiol production is mediated by the oocyte itself (Otsuka *et al.*, 2005). Oestradiol also exerts a paracrine action on theca cells to up-regulate LH-induced secretion of androgen, an essential requirement for further oestradiol production by the pre-ovulatory follicle. Production of inhibin, like oestradiol, increases in selected dominant follicles in an FSH- and IGF-dependent manner (Gutierrez *et al.*, 1997; Fujiwara *et al.*, 2001b; Spicer *et al.*, 2002). P450 aromatase activity also increases progressively, reaching very high levels in the granulosa cells of the preovulatory follicle. From primary follicle stage onwards, granulosa cells express 17 β -hydroxysteroid dehydrogenase (17 β -HSD), but co-expression of both P450arom and 17 β -HSD enables granulosa cells to become highly active in converting theca-derived androstenedione to oestradiol (Figure 1.2).

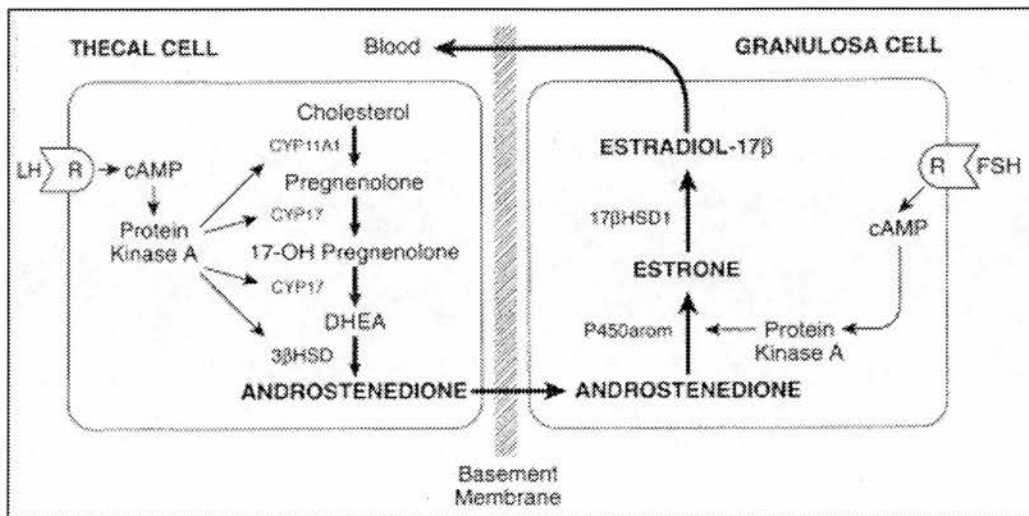


Figure 1.2 Schematic diagram of the ‘two cell, two gonadotrophin’ model of regulation of oestrogen synthesis.

Luteinising hormone receptors are located on theca cells during all stages of the menstrual cycle. The CYP17 gene encoding 17-hydroxylase/C17-20-lyase activity is crucial to androgen synthesis, and is expressed exclusively in thecal cells. LH principally stimulates androstenedione production in the theca cells. Androstenedione is then transported to the granulosa cells where it is aromatized to oestrone and finally converted to oestradiol.

FSH receptors exist exclusively on the granulosa cell membranes. FSH can act directly to induce cytoproliferation and differentiation via cAMP/PKA pathway. Increased FSH levels during the late luteal phase results in induction of the CYP19 gene and expression of P450 aromatase, resulting in large increases in oestradiol secretion by granulosa cells.

In the mid - late follicular phase, rising systemic oestrogen levels negatively feedback and diminish FSH secretion from the anterior pituitary (and positively feedback to stimulate LH secretion). It is ultimately the fall in FSH that is the key to follicular dominance. The cohort of developing follicles is dependent on FSH for growth and development and the fall in FSH levels below threshold levels results in apoptosis of granulosa cells and atresia (Erickson and Shimasaki, 2001). In humans, usually only a single follicle will continue onward development. By definition this follicle has higher sensitivity to FSH, by virtue of increased receptor expression, although it is postulated that it may also act directly on smaller follicles in the cohort to reduce their sensitivity to gonadotrophins (Driancourt, 2001). Follicular dominance is re-enforced by at least two local factors: IGF-1, which stimulates LH receptor formation (Chase *et al.*, 1998) and vascular endothelial growth factor (VEGF), a potent theca-derived promoter of angiogenesis whose production is stimulated by LH (Garrido *et al.*, 1993).

Competence of the preovulatory follicle to respond to the inductive stimulus of the LH surge and undergo ovulation also requires expression of LH receptors in granulosa cells. FSH plays a vital role in LH receptor induction, but expression of LH receptors remains suppressed until late in the follicular phase of the cycle (Minegishi *et al.*, 1997). There is compelling evidence that it is the oocyte itself that inhibits the expression of granulosa LH receptors in the developing antral follicle by release of inhibitory factors until the onset of the preovulatory stage (Vanderhyden, 1993; Coskun *et al.*, 1995; Vanderhyden and Tonary, 1995; Li *et al.*, 2000). Shortly after the LH surge, the oocyte resumes

meiosis, and undergoes maturational cytoplasmic changes that prepare it for fertilisation. This will be considered in detail later.

In mammals, 99.9% of all the follicles (oocytes) die by atresia, a process that occurs by apoptosis in both oocyte and granulosa cells (Tilly, 1996). The molecular pathway of apoptosis was first worked out in *Caenorhabditis elegans* (Ellis and Horvitz, 1986; Metzstein *et al.*, 1998) but is well conserved across the species. In mammals, it involves the evolutionarily conserved Bcl-2 family of proteins, which are located in the mitochondrial membrane (Green and Reed, 1998). The Bcl-2 family is composed of both pro- and anti-apoptotic agents (Adams and Cory, 1998). The ratio between them regulates the caspase cascade, which leads to cell death when activated, and determines, in part, the susceptibility of cells to a death signal (Gross *et al.*, 1999; Vander Heiden and Thompson, 1999; Zhang *et al.*, 2000).

A variant FSH receptor has been identified in the ovary that can activate Ca^{2+} / protein kinase C (PKC) signalling activity (Babu *et al.*, 2000; Babu *et al.*, 2001). This altered form of FSH receptor is increased in ovaries of mice treated with pregnant mare serum gonadotrophin (PMSG). Incidence of follicular atresia is significantly reduced in antral follicles of ovaries in mammals treated with PMSG (Braw and Tsafiriri, 1980; Monniaux *et al.*, 1984) and it may be that follicular rescue is orchestrated via this modified FSH receptor. Physiological mechanisms governing atresia are outwith further discussion here.

1.5 OOCYTE MATURATION

Follicle enclosed oocytes are arrested in prophase of the first meiotic division. After a prolonged resting state, the oocyte in the preovulatory follicle resumes meiosis during the ovulation sequence. The oocyte nucleus, or germinal vesicle, undergoes a series of changes that involve germinal vesicle breakdown (GVBD), and progression of meiosis to metaphase II with extrusion of first polar body. The second meiotic division occurs without being preceded by DNA synthesis, and results in haploid germ cells. Meiosis is again arrested and proceeds no further unless the ovulated egg is fertilised.

Maturation of oocytes *in vivo* occurs after the preovulatory LH surge, and is concurrent with the final development of the dominant (preovulatory) follicle. The ability to initiate maturation is closely correlated with increasing follicle size (Trounson *et al.*, 2001) and requires a complex series of molecular and structural events. Granulosa gene expression switches from follicular growth to the requirements of ovulation and luteinisation (Richards, 1994; Richards *et al.*, 1998) and oocyte protein translation actively continues with accumulation of transcripts and macromolecules, in preparation for meiosis and early embryonic development (Wickramasinghe and Albertini, 1993; Fair *et al.*, 1995).

Oocyte maturation is defined by both nuclear and cytoplasmic maturation. Nuclear maturation involves resumption of meiosis. It encompasses condensation of chromosomes and GVBD, cell cycle progression through metaphase I, anaphase I and telophase I, ending in meiotic arrest at metaphase II, with alignment of chromosomes along the metaphase plate and extrusion of the first polar body (Trounson *et al.*, 2001).

Cytoplasmic maturation is a less easily defined process, but entails molecular and structural changes to provide a matured oocyte with the capacity to undergo fertilisation, embryo cleavage and development to blastocyst stage (Kastrop *et al.*, 1990).

Follicular cells, particularly cumulus-granulosa, are crucial to appropriate cytoplasmic maturation (Kim *et al.*, 1997; Hashimoto *et al.*, 1998; Tanghe *et al.*, 2002) and many endocrine and paracrine factors have been shown to affect maturation of oocytes, either directly or signalled via cumulus cells. Transient expression of progesterone receptor mRNA (Park and Mayo, 1991), and the prostaglandin-endoperoxide synthetase 2 gene (Ptgs2, also known as COX-2) (Sirois *et al.*, 1992; Sirois *et al.*, 1993) have been identified following the LH surge. It is possible that these genes may be involved in the ovulation process itself rather than oocyte maturation. However, increased progesterone production and induction of progesterone receptor expression in response to LH and FSH has been demonstrated in porcine cumulus cells *in vitro*. And inhibition of progesterone production using aminoglutethimide in the same experimental model resulted in inhibition of GVBD, an effect overcome by addition of progesterone (Shimada and Terada, 2002).

During the process of maturation cumulus cells undergo mucification followed by expansion. The onset of mucification is marked by a dramatic increase in the secretion of mucopolysaccharides into the extracellular spaces. This leads to the dispersal of cumulus cells and causes the oocyte-cumulus complex to expand. The process of cumulus expansion appears to be initiated by the preovulatory increase in FSH. Oocyte-

derived GDF9 and/or BMP15 may act on the cumulus granulosa cells to attenuate estradiol production and promote progesterone and hyaluronic acid production, and therefore mucification and cumulus expansion (Elvin *et al.*, 1999). Cumulus expansion is physiologically important not only because it is required for normal ovulation and fertilisation (Chen *et al.*, 1993; Talbot *et al.*, 2003), but also because disruption of oocyte-cumulus gap junctions results in termination of bi-directional communication between the oocyte and its surrounding somatic cells. Inhibition of hyaluronic acid synthesis or the covalent linking of cumulus cells *in vitro* results in marked reduction of ovulation rates (Chen *et al.*, 1993; Hess *et al.*, 1999).

1.6.1 NUCLEAR MATURATION

A complex cascade of phosphorylation and dephosphorylation events is involved in regulation of meiosis resumption. Activation of maturation promoting factor (MPF) is responsible for the onset of oocyte maturation and either precedes or occurs concomitantly with GVBD (Gordo *et al.*, 2001). MPF is a serine-threonine heterodimer composed of a catalytic subunit, p34^{cdc2} kinase, and a regulatory subunit, cyclin B (Labbe *et al.*, 1989; Pines and Hunter, 1989; Gautier *et al.*, 1990). Active MPF is able to phosphorylate proteins that form the nuclear envelope and those involved in chromatin condensation and cytoskeletal reorganisation (Moreno and Nurse, 1990). Cyclic MPF activity is characteristic of oocytes. MPF assembly and activation increases until metaphase I and decreases during anaphase to telophase transition (Wehrend and Meinecke, 2001). The activity increases again until the oocyte reaches metaphase II, and is maintained at a high level by the interaction of cytosstatic factor (CSF) and the viral

oncogene c-mos until fertilisation (Trounson *et al.*, 2001; Wehrend and Meinecke, 2001).

Mitogen-activated protein kinases (MAP kinases) are other serine-threonine kinases known to be involved in oocyte maturation. MAP kinase is activated at the onset of oocyte maturation by a protein kinase cascade (Haccard *et al.*, 1990; Verlhac *et al.*, 1993; Inoue *et al.*, 1995). In mammalian oocytes two isoforms of MAP kinase are present, known as extracellular-regulated kinase 1 and 2 (ERK1 and ERK2). MAP kinase activity is required for maintenance of MPF activity, regulation of microtubule dynamics, spindle assembly and maintenance of MII arrest (Verlhac *et al.*, 1993; Colledge *et al.*, 1994; Hashimoto *et al.*, 1994; Verlhac *et al.*, 1994; Dedieu *et al.*, 1996; Gordo *et al.*, 2001).

The level of cAMP in the oocyte plays a critical role in meiotic maturation. Spontaneous resumption of meiosis was originally noted to be reduced in mammalian oocytes where media was supplemented with cAMP (Jagiello *et al.*, 1975) and it is now accepted that meiotic arrest is maintained in vivo by high levels of cAMP in the oocyte, via the PKA pathway (Maller and Krebs, 1977; Maller and Krebs, 1980; Bornslaeger *et al.*, 1986; Bilodeau-Goeseels, 2003; Masciarelli *et al.*, 2004). Consistent with this hypothesis, pharmacological manipulation to increase cAMP levels in mammalian oocytes, including phosphodiesterase (PDE) inhibitors, blocks oocyte maturation both *in vitro* and *in vivo* (Urner *et al.*, 1983; Conti *et al.*, 1998; Wiersma *et al.*, 1998). FSH retards nuclear maturation via a cAMP-dependent pathway (Eppig *et al.*, 1983) but enhances

fertilisation and developmental ability of oocytes when supplemented to a bovine *in vitro* maturation system (Izadyar *et al.*, 1998; Tatemoto and Terada, 1998). In addition to the PKA pathway, protein kinase C may also be involved in meiotic regulation of oocyte maturation (Su *et al.*, 1999; Downs *et al.*, 2001).

The surge in LH activates adenylate cyclase activity and results in a rapid, but transient, rise in cAMP (Dekel *et al.*, 1988). As stated earlier, high concentrations of intra-oocyte cAMP inhibit meiotic maturation, and an oocyte will resume meiosis following removal of inhibitory actions of cAMP. It would seem, however, that a transient increase in cAMP concentration after the gonadotrophin surge is required to initiate oocyte maturation (Dekel *et al.*, 1988; Yoshimura *et al.*, 1992). Thereafter the level of cAMP in the oocyte falls abruptly and allows commitment to meiosis, possibly by desensitisation and down regulation of granulosa LH and FSH receptors or by activation of specific phosphodiesterases that degrade cAMP (Tsafriri *et al.*, 1996; Conti *et al.*, 2002; Mayes and Sirard, 2002). The LH surge also stimulates rapid increases in intracellular calcium in granulosa and cumulus cells, via cAMP (Webb *et al.*, 2002). Activation of phospholipase C (PLC) also results in increased levels of IP3 in both granulosa and oocyte, and raises intraoocyte calcium as a result of extracellular influx as well as intracellular calcium mobilisation (Homa, 1995). Transient increases in intracellular calcium and modification of proteins (phosphorylation, dephosphorylation or activation of proteases) are responsible for driving the meiotic cell cycle through various control points. This includes synthesis and modification of cyclins, resulting in activation of MPF and GVBD.

1.6.2 CYTOPLASMIC MATURATION

Cytoplasmic maturation is difficult to define. It encompasses changes in distribution and organisation of the individual organelles, migration of mitochondria to perinuclear positions and accumulation of cortical granules along the oolemma. These changes allow fertilisation to occur and include acquisition of abilities to decondense penetrated spermatozoa, form a block to polyspermy and to form pronuclei after fertilisation. Most oocytes that have achieved nuclear maturation are capable of responding to sperm to form a zygote, but far fewer zygotes develop to blastocyst stage *in vitro*. Another aspect of cytoplasmic maturation involves the transcription and translation of specific mRNA in order to acquire embryonic developmental potential. Deficient mRNA storage during oocyte maturation results in lower developmental competence (Leoni *et al.*, 2007).

Fertilisation initiates a cascade of cellular events required for embryo and fetal development. However, the earliest stages of development are largely dependent on maternally derived messages stored in the oocyte prior to fertilisation. A mature mouse oocyte contains approximately 200-fold more RNA and 50 – 60-fold more protein than an average somatic cell (Wassarman and Kinloch, 1992). In addition, the proportion of mRNA is 15 - 20%, compared to 2 – 3% in a somatic cell (Wassarman and Kinloch, 1992). RNA transcribed during cytoplasmic maturation is very stable. Different degrees of polyadenylation define transcripts for immediate use, or for storage and utilisation following elongation of the poly-A tail (Bachvarova, 1992). Active transcription ceases with resumption of meiosis, but translation of the mRNA pool continues throughout the final stages of meiosis and beyond (Kinloch *et al.*, 1993). As embryo development progresses, maternal (oocyte) RNAs and proteins are depleted and embryo-derived

messages become the key controlling factors. The shift from dependence on oocyte-derived messages to embryo-produced messages is referred to as maternal to embryonic transition (MET) and occurs at different stages in a species dependent manner: 2-cell stage in mice, 4- to 8-cell stage in humans and 8- to 16-cell stage in cattle (Telford *et al.*, 1990; Memili and First, 2000). Early embryo development, therefore, is the ultimate manifestation of appropriate cytoplasmic maturation.

1.7 *IN VITRO* MATURATION

Mammalian oocytes are able to mature *in vitro* when they are isolated from follicles and placed in appropriate medium (Pincus and Enzmann, 1935). *In vitro* maturation (IVM) of mammalian oocytes, including human, was initially studied and reported by Edwards in 1965 (Edwards, 1965b; Edwards, 1965a) and offered a source of oocytes for *in vitro* fertilisation (IVF). Hormonal stimulation has proved to be a more effective means of obtaining oocytes, but carries implications, both in terms of the financial expense of assisted reproduction cycles as well as the risk of potentially serious medical complications such as ovarian hyperstimulation. IVM of oocytes offers an alternative to the use of high levels of exogenous gonadotrophins and potentially stands to benefit patients, particularly those suffering from polycystic ovary syndrome (PCOS), who may be extremely sensitive to stimulation with exogenous gonadotrophins, or women with normal ovaries and regular cycles who are referred for IVF due to male factor infertility. As well as the clinical setting, this technology would also be of benefit to agricultural breeding programmes. The clinical feasibility of assisted reproduction using immature oocytes in humans was realised in the early 1990s (Cha *et al.*, 1991; Trounson *et al.*,

1994), but early reports of clinical pregnancies had unacceptably low success rates (Cha *et al.*, 1991; Trounson *et al.*, 1994; Russell *et al.*, 1997). Developmental faults of *in vitro* matured oocytes and poor pregnancy outcomes are most likely to be due to impaired cytoplasmic maturation, and much research effort has, as a result, been directed at optimising IVM culture conditions. Improvement in methods and technology has resulted in increasing clinical successes, with recent reported pregnancy rates of 20 - 24% (Le Du *et al.*, 2005; Li *et al.*, 2005; Mikkelsen, 2005), up to 38% per cycle (Rao and Tan, 2005). The few children born following IVM thus far appear to be healthy (Mikkelsen, 2005).

Oocytes in culture are affected by the physical conditions of the *in vitro* environment, specifically osmolarity (Yamauchi *et al.*, 1999), ionic composition, temperature (Shi *et al.*, 1998), pH (Downs and Mastropolo, 1997), CO₂ (Geshi *et al.*, 1999) and O₂ tension (Takahashi *et al.*, 1996), as well as culture volume. Most researchers agree that impaired cytoplasmic maturation can be overcome by optimisation of culture conditions (Cha and Chian, 1998). Addition of various hormones, oestrus maternal serum or follicular cells to culture medium has been shown to be beneficial for IVM, fertilisation and development of immature follicular oocytes in mammals (Younis *et al.*, 1989; Armstrong *et al.*, 1991; Ali and Sirard, 2002). Estradiol has also been shown to support cytoplasmic maturation changes necessary for fertilisation and early post-fertilisation development (Tesarik and Mendoza, 1995; Guler *et al.*, 2000). Commonly used supplements, such as fetal serum and serum albumin, are known to be beneficial to *in vitro* oocyte maturation and embryo development but contain numerous factors including growth factors, lipids, albumin,

hormones, steroids, cholesterol, peptides and, indeed, many undefined substances (Gardner and Lane, 1993). The highly undefined nature of these supplements, as well as risk of batch variation, makes them undesirable for research or clinical practice. High-grade bovine serum albumin (BSA) also carries some degree of variability, but is less variable than serum itself (Sutton *et al.*, 2003). BSA has also been shown to contain steroids, notably estradiol, at levels high enough to allow for adequate cytoplasmic and nuclear maturation such that supplementation with estradiol is unnecessary (Mingoti *et al.*, 2002). Synthetic macromolecules, such as polyvinyl alcohol, have been used in place of serum to standardise culture media (Pinyopummintr and Bavister, 1991; Keskinetepe and Brackett, 1996) and some studies in animals have demonstrated that serum-free conditions can support oocyte maturation *in vitro* (Eppig *et al.*, 1992; Serta *et al.*, 1995). This carries important advantages in terms of safety and quality control of culture media.

Many have investigated roles for the myriad of growth factors expressed in developing follicles, or whose receptors are expressed, and/or that stimulate granulosa and thecal cell proliferation and differentiation, by their addition to serum-free maturation systems (Gougeon, 1996). Growth factors beneficial to IVM include those which improve maturation by a direct effect on oocytes and enhancement of nuclear maturation, such as activin A, as well as those where maturation appears to improve indirectly as a result of beneficial effects mediated by cumulus, such as epidermal growth factor (EGF). These are now considered further.

Activins and inhibins are produced by granulosa cells and act on both oocytes and granulosa in a paracrine / autocrine manner, either directly or via gap junctions. All inhibin / activin subunits and receptor mRNA transcripts are present in oocytes and granulosa cells (Izadyar *et al.*, 1998; Sidis *et al.*, 1998; Silva *et al.*, 2003) and qualitative and quantitative changes in expression and distribution of inhibin and activin subunits in bovine oocytes have been reported during IVM and fertilisation (Izadyar *et al.*, 1998; Silva *et al.*, 2003). The effect of activin A on meiotic maturation was originally investigated in oocytes from immature rats treated with PMSG (Itoh *et al.*, 1990), which found it accelerated maturation of not only follicle-enclosed oocytes and oocyte-cumulus complexes, but also denuded oocytes, as measured by an increase in the percentage of oocytes with GVBD. Oocyte maturation is unaffected by activin A in the presence of an inhibitor of GVBD such as cAMP (Itoh *et al.*, 1990), and the effect of activin A is also inhibited by its binding protein follistatin (Sadatsuki *et al.*, 1993; Alak *et al.*, 1996). Activin A is a proven potent stimulator of primate and human oocyte maturation *in vitro* (Alak *et al.*, 1996; Alak *et al.*, 1998) and has also been shown to promote maturation and developmental competence, measured by significantly enhanced postcleavage development (Stock *et al.*, 1997; Silva and Knight, 1998).

Human oocytes express EGF and its receptor (Maruo *et al.*, 1993; Bennett *et al.*, 1996). It is also found in follicular fluid (Artini *et al.*, 1994). A number of studies have implicated EGF in growth and differentiation of the ovarian follicle and steroidogenesis (Mondschein and Schomberg, 1981; Schomberg *et al.*, 1983), and it is known that purified EGF induces GVBD and nuclear maturation when added to cumulus-oocyte

complexes or to intact follicles in culture (Dekel and Sherizly, 1985; Downs *et al.*, 1988; Downs, 1989). EGF can induce cumulus expansion as efficiently as FSH (Harper and Brackett, 1993; Lonergan *et al.*, 1996) and enhances nuclear maturation, an effect mediated by cumulus cells and promoted by gonadotrophins (Lorenzo *et al.*, 1994; Wang and Niwa, 1995; Lonergan *et al.*, 1996; Goud *et al.*, 1998; De La Fuente *et al.*, 1999; Guler *et al.*, 2000; Lorenzo *et al.*, 2002; Kim *et al.*, 2004; Kishida *et al.*, 2004). EGF has been shown to promote steroidogenesis in two different *in vitro* models of oocyte-granulosa cell complexes (Jamnongjit *et al.*, 2005), and it may be that steroid production represents one pathway by which EGF receptor signalling promotes oocyte maturation in gonadotropin-stimulated follicles. EGF also increases the developmental potential of *in vitro* matured oocytes in terms of fertilisation (Goud *et al.*, 1998; Illera *et al.*, 1998; Kishida *et al.*, 2004) and / or development to blastocyst stage (Harper and Brackett, 1993; Lonergan *et al.*, 1996; Abeydeera *et al.*, 1998; De La Fuente *et al.*, 1999; Guler *et al.*, 2000; Merlo *et al.*, 2005).

1.8 FERTILISATION / PARTHENOGENETIC ACTIVATION

Bovine oocytes undergo embryo development up to the blastocyst stage after parthenogenetic activation (Wang *et al.*, 1999) with the same efficiency yielded by IVF (Boni *et al.*, 2002). However, mammalian parthenogenetic embryos show different characteristics to IVF embryos (Winger *et al.*, 1997) and fail to generate viable offspring (Fukui *et al.*, 1992). Parthenogenetic activation and fertilisation initially share common mechanisms; both show electrical responses and calcium oscillations (Nuccitelli, 1980; Schlichter and Elinson, 1981; Swann, 1990; Dale *et al.*, 1996). One of the first events

occurring at fertilisation, or parthenogenetic activation, is a transient modification of the electrical properties of the oocyte plasma membrane potential. The electrical events following fertilisation or parthenogenetic activation are generated by potassium currents in mammals, and may be calcium-activated (Miyazaki and Igusa, 1982; Dale *et al.*, 1996) or calcium-independent (Igusa *et al.*, 1983). The potassium ion current results in hyperpolarisation of the plasma membrane (Gianaroli *et al.*, 1994; Tosti *et al.*, 2002). Fertilisation triggers a wave of oscillations in the intracellular concentrations of calcium, which has been characterised in human oocytes following IVF (Taylor, 1994; Tesarik *et al.*, 1995) as well as intracytoplasmic sperm injection (ICSI) (Tesarik, 1994). Repetitive calcium oscillations follow the initial wave of calcium release up to extrusion of the second polar body (Russo *et al.*, 1996; Yoshida *et al.*, 1998) and pronuclear formation (Stricker, 1999; Swann and Parrington, 1999). Calcium is derived from intracellular stores, which may increase during oocyte maturation (Jones *et al.*, 1995) and is supplemented by extracellular calcium influx through the plasma membrane.

Mature oocytes can be artificially activated by a number of different methods: 1) addition of chemicals to increase intracellular calcium content, including calcium ionophores such as ionomycin (Steinhardt and Epel, 1974) or calcium-dependent adenosine triphosphatase (ATPase) inhibitors such as cyclopiazonic acid and thapsigargin (Petr *et al.*, 2000), 2) ethanol treatment (Nagai, 1987), which increases conductance of calcium-activated K^+ -channels (Dopico *et al.*, 1996), 3) electric shock (Collas *et al.*, 1993), or 4) caged chelators, which release intra-oocyte calcium in response to specific wavelengths. The basis behind these various methodologies is to

mediate extracellular influx and/or intracellular release of free calcium into the oocyte cytoplasm. The calcium transient is believed to be the upstream event that triggers the cascade of cellular changes necessary for resumption of meiosis and the cell cycle. Only the initial calcium surge induced by ionophore, electric shock or other method is required, as a calcium-induced transient will continue in a self-perpetuating cycle. Magnesium or other divalent cations, such as strontium and barium may also be supplemented to the medium and oocyte in lieu of calcium. Increases in intraoocyte calcium results in inactivation of MPF, and subsequently CSF. Another method of parthenogenetic activation is targeted at inhibiting protein synthesis using cycloheximide (Presicce and Yang, 1994b; Liu *et al.*, 1998), and / or reduction of phosphorylation of cellular proteins such as MPF, CSF and cytoskeletal proteins using 6-dimethylaminopurine (DMAP), a serine-threonine kinase inhibitor (Neant and Guerrier, 1988). However, agents that increase intracellular calcium or inhibitors of protein synthesis or phosphorylation alone result in low cleavage and development rates (Liu *et al.*, 1998). Synergistic effects on activation have been reported in the bovine model by combining agents that increase intracellular calcium with inhibitors of protein synthesis / phosphorylation (Presicce and Yang, 1994b; Presicce and Yang, 1994a; Liu *et al.*, 1998), with optimal bovine parthenogenetic development using a calcium ionophore followed by incubation in either DMAP or cycloheximide and cytochalasin B (Liu *et al.*, 1998). This method of parthenogenetic activation (ionomycin, followed by co-incubation with DMAP and cycloheximide) was used in the experiments performed in chapter 5.



1.9 TRANSFORMING GROWTH FACTOR β (TGF β FAMILY)

The TGF β superfamily is comprised of >35 proteins that share structural similarity (Chang *et al.*, 2002). These proteins are synthesised as large precursor proteins that are proteolytically cleaved to yield the mature protein and include members of the TGF β , activin / inhibin, GDF and BMP subfamilies as well as proteins such as AMH. They are composed of an amino-terminal signal sequence, a variable pro-domain and a highly conserved carboxyl-terminal domain. The signalling part of the molecule is the carboxyl-terminal domain, which generally contains seven conserved cysteine residues. Six of these cysteines form three disulphide bonds within each monomer, which interact to build a structure termed the cysteine knot. This knot contributes to the stabilisation of various conformations of TGF β superfamily members. The remaining cysteine residue in each monomer forms an additional disulphide bond, and links two monomers into a dimer. This cysteine is missing in some members, for example GDF9 and BMP15, but multiple hydrophobic contacts between two monomer subunits promote dimerisation, even in the absence of a disulphide bond (Kingsley, 1994).

1.10 INHIBINS AND ACTIVINS

Inhibins and activins are structurally related gonadal proteins originally identified by their ability to alter FSH secretion from the pituitary (Vale *et al.*, 1986; Vale *et al.*, 1988). Inhibin selectively suppresses FSH secretion (Vale *et al.*, 1988), whereas activin stimulates FSH release (Ling *et al.*, 1986; Schwall *et al.*, 1989). Apart from their action on FSH secretion, inhibins and activins have been shown to exert paracrine / autocrine

effects within the gonads (Lin *et al.*, 1989; Chen, 1993) and many other tissues (Spencer *et al.*, 1990) and have also been proposed to have important paracrine function(s) during fetal development (Albano *et al.*, 1994). Indeed, many members of the TGF β superfamily are pivotal in controlling cellular growth and differentiation during fetal, as well as adult life (Heldin *et al.*, 1997; ten Dijke *et al.*, 2000).

Inhibins are composed of one α subunit and one of two similar but distinct β subunits (βA and βB). Activins are dimers of two β subunits, namely activin A ($\beta A/\beta A$), activin B ($\beta B/\beta B$) and activin AB ($\beta A/\beta B$) (Vale *et al.*, 1988). Two further mammalian activin subunits have also been cloned, βC (Schmitt *et al.*, 1996) and βE (Fang *et al.*, 1996). They are mainly derived from hepatic production (Vejda *et al.*, 2002), but their functions are yet to be determined. Activin was originally isolated from gonadal sources and was assumed, like inhibin, to have endocrine actions. It is now accepted to predominantly act as a local growth and differentiation factor, on the basis of distribution of activin and its receptors in various tissues, as well as the fact that circulating activin appears to be bound almost irreversibly to follistatin (Schneyer *et al.*, 1994).

1.11 FOLLISTATIN

Follistatins are cysteine-rich (36 cysteine residues) glycoproteins, structurally unrelated to inhibins but reported to have the ability to suppress FSH secretion (Robertson *et al.*, 1987; Ueno *et al.*, 1987). They bind activins with high affinity (Kogawa *et al.*, 1991a; Schneyer *et al.*, 1994). To a lesser extent, they also bind inhibins, because of the

common β subunit, (Shimonaka *et al.*, 1991) but do not bind to activin receptors.

Follistatin is a natural antagonist to the biological activities of activin (Nakamura *et al.*, 1990; Kogawa *et al.*, 1991b) and is believed to act primarily in an autocrine / paracrine manner to modulate its availability and bioactivity. Follistatin is found in the pituitary, testis (Kogawa *et al.*, 1991b) and ovary (Nakamura *et al.*, 1990; Nakamura *et al.*, 1991) as well as a variety of other tissues, suggesting widespread regulation of the various biological actions of activin. Follistatin has also been observed to bind other TGF β superfamily members, including several BMPs and certain members of the GDF family (Yamashita *et al.*, 1995; Fainsod *et al.*, 1997; Iemura *et al.*, 1998; Gamer *et al.*, 1999; Otsuka *et al.*, 2001).

Follistatin is synthesised as a precursor of 344 amino acids (Shimasaki *et al.*, 1988), and exists as a variety of isoforms. Two variants of follistatin are encoded through alternative splicing at the carboxyl terminus: a 288-residue molecule (FS-288) and a 315-residue form (FS-315), which is extended by an extra exon to include a highly acidic tail. Both are capable of binding activin, although FS-288 may bind with higher affinity, and therefore confer higher activin-neutralising activity, than FS-315 (Inouye *et al.*, 1991; Hashimoto *et al.*, 2000). Secreted FS-315 may be further processed by enzymatic cleavage to shorter intermediate forms of 300 – 303 residues (FS-300/303), which are predominantly localised to ovarian follicles (Inouye *et al.*, 1991; Sugino *et al.*, 1993). Serum and follicular follistatin are biochemically distinct (Schneyer *et al.*, 1996). Nearly all circulating follistatin is full-length (Khoury *et al.*, 1995; Macias-Silva *et al.*,

1996) and bound to activin (Schneyer *et al.*, 1994; McConnell *et al.*, 1998). Whether it has any physiological or pathophysiological significance remains to be determined.

1.12 ACTIVIN / INHIBIN RECEPTORS

Members of the TGF β superfamily use a common mechanism to signal to the cell nucleus. They bind two types of closely related membrane receptors (designated type I and type II), which have intracytoplasmic serine-threonine kinase domains. Presently seven type I receptors (activin receptor-like kinase (ALK) 1 – 7) and five type II receptors (ActRIIA, ActRIIB, BMPRII, TGF β RII and AMHRII) have been identified (de Caestecker, 2004; ten Dijke and Hill, 2004). Type I and type II receptors are glycoproteins of approximately 55kDa and 70 kDa respectively. Activin signals through type I and type II receptors each represented by two isoforms: ALK2 / ALK4 and ActRIIA / ActRIIB respectively, which interact upon ligand binding (Ebner *et al.*, 1993; ten Dijke *et al.*, 2000). Activin binds a type II receptor first, which then facilitates binding of a type I receptor. In contrast to BMP receptors, only activin-bound type II receptors physically interact with type I receptors (Ebner *et al.*, 1993; Franzen *et al.*, 1993; Bassing *et al.*, 1994; Attisano *et al.*, 1996). Ligand binding leads to assembly of a heterotetrameric receptor complex, which consists of two type II receptors and two type I receptors (Macias-Silva *et al.*, 1996; Massagué, 1998). Association of type I and type II receptors is a requirement for mediation of the biological actions of activin (Carcamo *et al.*, 1994). The receptor complex mediates signalling by phosphorylating proteins of the Smad family (Heldin *et al.*, 1997; Massagué, 2000). There are 8 Smad proteins in

total, designated Smad 1 – 8. Members of the TGF β / activin subfamilies typically activate Smad 2 and 3 (Baker and Harland, 1996; Chen *et al.*, 1996; Macias-Silva *et al.*, 1996; Zhang *et al.*, 1996) whereas Smad 1,5 and 8 are activated by members of the BMP subfamilies (Graff *et al.*, 1996; Hoodless *et al.*, 1996; Liu *et al.*, 1996; Chen *et al.*, 1997; Yamamoto *et al.*, 1997). In an inactive (non-phosphorylated) state, Smad 2 and Smad 3 exist as monomers (Kawabata *et al.*, 1998). A membrane-associated protein, Smad anchor for activation (SARA), controls Smad recruitment to the activin receptor complex (Tsukazaki *et al.*, 1998). SARA presents Smad 2 / Smad 3 to the activated type I receptor by binding non-phosphorylated Smads as well as binding the receptor complex. Once phosphorylated, Smad 2 / Smad 3 dissociates from SARA and the activin receptor complex and combines with Smad 4, the common Smad. The resultant hetero-oligomers (Kawabata *et al.*, 1998) then translocate to the nucleus where they regulate transcription of gene targets (Massagué, 1998; Massague and Wotton, 2000). Smads 6 and 7 are inhibitory Smads that prevent signalling pathway activation (Imamura *et al.*, 1997; Bhushan *et al.*, 1998; Ishisaki *et al.*, 1998; Nakayama *et al.*, 1998; Liu *et al.*, 2002b). Smad proteins are well documented as the central elements in activin receptor signalling, but may not represent the only pathway activated by this receptor complex. The Smad signalling system is also modulated by crosstalk with other kinase signalling cascades (Derynck *et al.*, 1998; Massague and Wotton, 2000; Zimmerman and Padgett, 2000) and activin has been shown to activate the p38 MAPK pathway (Cocolakis *et al.*, 2001) (Figure 1.3).

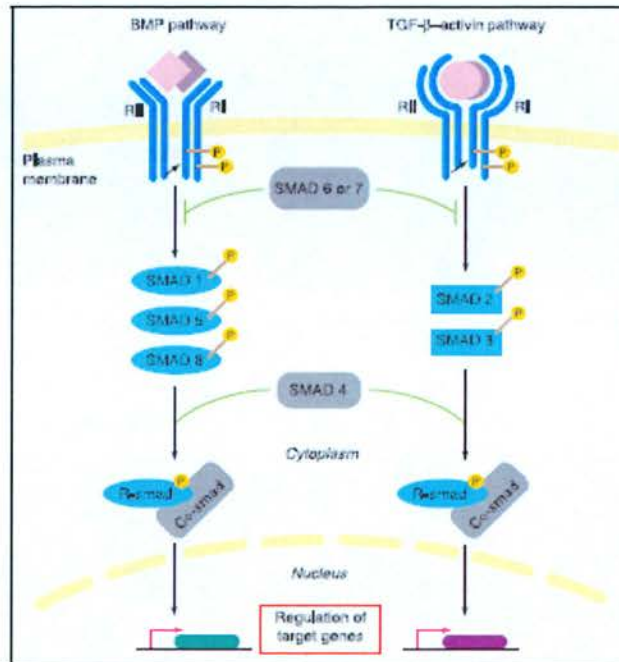


Figure 1.3 Diagram illustrating signalling pathways for BMP and TGF- β / activin.

Activin binding leads to assembly of a heterotetrameric complex, consisting of two type II and two type I receptors. The receptor complex then phosphorylates proteins of the Smad family (Smad 2 or Smad 3). The common Smad, Smad 4, combines with Smad 2/3 and regulates gene transcription once translocated to the cell nucleus. Inhibitory Smads 6 and 7 prevent signalling pathway activation (Lin *et al.*, 2003).

A third receptor type, TGF β RIII, or betaglycan, has also been identified. Betaglycan is a membrane-anchored proteoglycan that can bind inhibin and facilitate its association with activin type II receptors, thereby antagonising the actions of activin (Gray *et al.*, 2002) and potentially a number of BMPs (Wiater and Vale, 2003).

1.13 INHIBINS AND ACTIVINS IN THE OVARY

Extensive literature exists documenting expression and roles of inhibins, activins and follistatin in adult mammalian ovarian function. Inhibin / activin subunits are differentially expressed during folliculogenesis (Schwall *et al.*, 1990; McNatty *et al.*, 1999), and have been demonstrated to regulate growth and development of ovarian follicles (Woodruff *et al.*, 1990; Findlay, 1993; Li *et al.*, 1995) as well as oocyte maturation (Itoh *et al.*, 1990; Alak *et al.*, 1996; Alak *et al.*, 1998) (Figure 1.4).

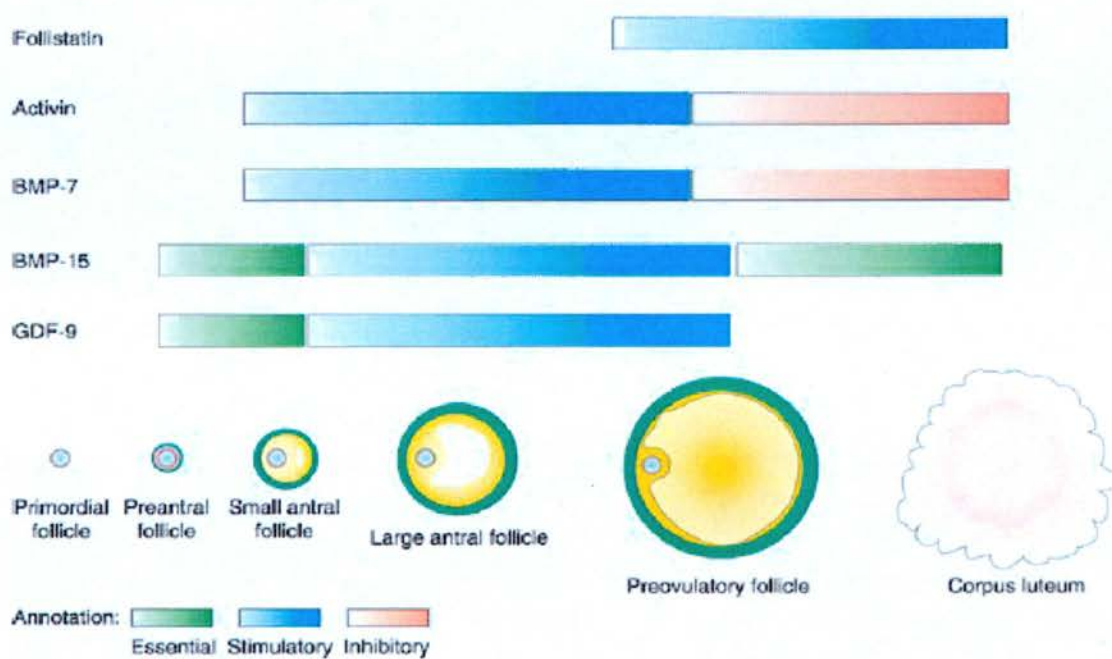


Figure 1.4 Diagram illustrating involvement and effects of TGFβ superfamily members and follistatin during folliculogenesis (Lin *et al.*, 2003).

Multiple mutant mouse lines have been generated to facilitate study of mammalian reproduction and development and the associated roles of TGF β superfamily members *in vivo*. Female knockout mice lacking the inhibin α subunit (*Inha*^{-/-}) are depleted of the biological effects of inhibins and have increased FSH concentrations. They develop steroidogenic sex-cord stromal tumours of granulosa cell lineage with an associated cachexia-like wasting syndrome and typically die by 18 weeks of age (Matzuk *et al.*, 1992; Matzuk *et al.*, 1994). High levels of FSH (and LH) would appear to be critical to the process of tumour development; double mutant mice homozygous for the hypogonadal null mutation in the gonadotrophin releasing hormone (GnRH) gene (*hpg*) and the *Inha* knockout allele do not develop tumours (Kumar *et al.*, 1996), and *Fshb* and *Inha* double knockouts develop tumours with later onset and a less aggressive course (Kumar *et al.*, 1999). Inhibins are also thought to act independently as tumour suppressors (Pierson *et al.*, 2000). Superovulation experiments in younger *Inha*^{-/-} females indicate that there are defects in late stages of follicle development (Matzuk *et al.*, 1996). In contrast, female mice that over-express inhibin α have reduced FSH and elevated LH levels. Females are also subfertile, due to a decrease in the number of ovulated oocytes, a defect that can be corrected by provision of exogenous gonadotrophins (Pierson *et al.*, 2000; Cho *et al.*, 2001).

Mice with a null mutation engineered at the activin / inhibin β A locus (*Inhba*^{-/-}) die neonatally due to craniofacial defects (cleft palate and loss of whiskers, upper incisors, lower incisors and lower molars) that prevent suckling (Matzuk *et al.*, 1995a; Matzuk *et*

al., 1995b; Ferguson *et al.*, 1998). Knockout mice lacking a functional β B locus (*Inhbb*^{-/-}) have developmental defects in eyelid closure, prolonged gestation, and a failure of mothers to nurse their litters (Vassalli *et al.*, 1994; Matzuk *et al.*, 1996). Mice deficient in both activin β A and activin β B develop to term with open eyelids and defects in whiskers, incisors and secondary palate formation (Matzuk *et al.*, 1995a; Matzuk *et al.*, 1995b). The additive defects seen in double homozygous mutants suggest that activin β A and activin β B have discrete developmental functions are unable to compensate for the loss of each other. The *Inhba*^{-/-} phenotype can, however, be rescued by replacing the activin / inhibin β A coding sequence with that of the activin / inhibin β B gene. Knock-in mice avoid craniofacial malformations and neonatal lethality, but females demonstrate enlarged external genitalia, hypogonadism and severely compromised fertility, demonstrating that activin A is an important ovarian growth factor. Ovaries of mice homozygous for the knock-in allele contain follicles at all stages of differentiation, but a substantially reduced number of pre-ovulatory follicles. Ovaries of mice hemizygous for the knock-in allele are smaller than controls and contain fewer large pre-antral follicles (Brown *et al.*, 2000). Serum FSH levels in these mice are slightly elevated, although whether this reflects loss of pituitary inhibin signalling, enhanced activin B signalling or is simply secondary to gonadal defects remains to be elucidated (Burns and Matzuk, 2002).

Knockout mice lacking activin receptor type II (ActRII) have suppressed pituitary and serum FSH levels. LH levels are unaffected. A small proportion of these mutant mice

have skeletal and facial abnormalities reminiscent of Pierre-Robin Syndrome in humans, but most lack these defects and develop into adults. Mutant ActRII mice exhibit gonadal pathologies, including infertility, undeveloped uteri, and developmental block at the antral follicle stage with associated follicular atresia and reduced number of corpora lutea. The significance of these findings is difficult to interpret as the phenotype may represent lack of FSH rather than absence of activin receptor signalling (Matzuk *et al.*, 1995b; Matzuk, 2000). Null mutations for activin type I receptors ALK2 or ALK4 are embryo lethal. Formation of the primitive streak and mesodermal cells is completely blocked in knockout mice lacking ALK4 (Gu *et al.*, 1998). Embryos homozygous for ALK2 mutation are arrested at early gastrulation, with abnormal visceral endoderm morphology and severe disruption of mesoderm formation (Mishina *et al.*, 1995; Gu *et al.*, 1999b).

Understanding of inhibin and activin fetal expression and their function during development remains less than complete. Expression of mRNA and protein for activin subunits and receptors has been described in fetal primate and human ovaries (Rabinovici *et al.*, 1991; Harkness and Baird, 1997; Billiar *et al.*, 2003). High interspecies conservation of activins, inhibins and activin receptors and the universal presence of activins in mammals, birds, amphibians and fish suggest an evolutionary conserved role of these proteins in animal development. Activin is essential to mesoderm induction in early *Xenopus* development (Dyson and Gurdon, 1997) and may play a role in regulation of morphogenesis during mammalian organogenesis (Ritvos *et*

al., 1995). In rat fetal gonad culture experiments, Activin A increases ovarian, but not testicular, gonadal and mesonephric cell proliferation (Kaipia *et al.*, 1994).

Chapter 3 describes experiments conducted to further define expression and localisation of activin subunits and receptors in the human fetal ovary during the mid-trimester. The effect of activin A on germ cell survival and proliferation was investigated using an *in vitro* tissue culture model.

1.14 NEUROTROPHINS

Neurotrophins are vertebrate-specific growth factors originally identified in the nervous system and recognised for their ability to support neuronal differentiation, maturation and survival (Henderson *et al.*, 1993; Davies, 1994; Snider, 1994). The original neurotrophin hypothesis states that the survival of developing neurons depends on a supply of neurotrophic factor, which is synthesised in the target field and in limiting amounts (Levi-Montalcini and Angeletti, 1968; Thoenen and Barde, 1980).

Neurotrophin synthesis commences with the arrival of the earliest axons in the peripheral target fields of sensory and sympathetic neurons (Davies, 1987; Korsching and Thoenen, 1988). The level of neurotrophin present is proportional to the final innervation density; high levels in future densely innervated areas and low levels in future sparsely innervated areas (Harper and Davies, 1990). After uptake by sensory and sympathetic fibres in their target fields, neurotrophins are conveyed by fast axonal transport to the cell bodies of the innervating neurons where they exert survival-

promoting effects (Hendry *et al.*, 1974a; Hendry *et al.*, 1974b; Korsching and Thoenen, 1983).

The most extensively studied group of nerve growth factors are members of the neurotrophin family. Four members of the neurotrophin family have been identified in mammals, nerve growth factor, brain derived neurotrophic factor, neurotrophin-3 and neurotrophin-4 (also known as neurotrophin-5) (Lindsay, 1996). All four neurotrophins share similar primary, secondary, tertiary and quaternary structures (the latter as homodimers). They mediate their biological actions by binding and activating two different receptor classes, the tropomyosin-related kinase (Trk) family of receptor tyrosine kinases (Trk A, TrkB and TrkC) (Kaplan *et al.*, 1991), and the p75 receptor, a member of the tumour necrosis factor (TNF) receptor superfamily. Individual Trk receptors have distinct ligand selectivity and cellular expression. TrkA preferentially binds NGF (Klein *et al.*, 1991a), TrkB binds BDNF and NT4 (Berkemeier *et al.*, 1991; Klein *et al.*, 1991b; Klein *et al.*, 1992) and TrkC binds NT3 (Lamballe *et al.*, 1991). NT3 is also capable of binding to and signalling through TrkA and TrkB at high concentrations.

BDNF and NT4 bind to the TrkB and p75 receptors with equal affinity (Klein *et al.*, 1991b; Klein *et al.*, 1992; Chao, 1994) and exhibit similar biological activities in many *in vitro* assays (Davies *et al.*, 1993; Ibanez, 1996). BDNF was identified in 1989 (Leibrock *et al.*, 1989). It was the second neurotrophin cloned, and was linked to NGF on the basis of similarities in mRNA and protein sequence. NT4 was originally

identified in *Xenopus* and viper (Hallbook *et al.*, 1991) while NT5 was first isolated from rat (Berkemeier *et al.*, 1991). Ip *et al* defined the structure of NT4 and described its chromosomal location, tissue distribution and receptor specificity (Ip *et al.*, 1992). Phylogeny and biological data have since shown NT4 and NT5 to be orthologues (Ip *et al.*, 1993). Thus, mammalian NT4 may also be known as NT5 or NT4/5.

Generation of strains of transgenic mice deficient for various neurotrophins has helped define their roles. NT4^{-/-} mice are long-lived and fertile and were originally thought to show no neurological deficit (Conover *et al.*, 1995). More recently, neurological defects have been described, including deficient taste bud formation (Liebl *et al.*, 1999) and impaired long-term memory (Xie *et al.*, 2000). In contrast, BDNF^{-/-} mice die postnatally. BDNF heterozygous mice are viable and fertile, but lower levels of BDNF result in effects on eating and social behaviour, specifically hyperphagia accompanied by significant weight gain in early adulthood and increased aggressiveness. These behavioural abnormalities are known to correlate with 5-HT dysfunction (Lyons *et al.*, 1999). Targeted mutation of the BDNF locus also results in severe deficiencies in coordination and balance, associated with excessive degeneration in several sensory ganglia including the vestibular ganglion (Ernfors *et al.*, 1994). Slowly adapting mechanoreceptors, but not other types of cutaneous afferents, require BDNF in postnatal life for normal mechanotransduction. The neurons lacking BDNF do not die, but instead show a profound and specific reduction in their mechanical sensitivity, an effect that can be rescued by postnatal treatment with recombinant BDNF (Carroll *et al.*, 1998). Mice lacking BDNF also suffer from specific defects of cardiac vasculature (Donovan *et al.*,

2000). The contrasting phenotypes of the BDNF- and NT4-deficient mice is believed to be a result of divergent expression of the two neurotrophins rather than a difference in biological action of the ligands *in vivo*, a theory supported by the rescue of the BDNF-deficient phenotype in mice where NT4 expression is knocked into the BDNF gene locus (Fan *et al.*, 2000).

Neurotrophins are synthesised as precursor pro-neurotrophins, which are then proteolytically cleaved to form mature neurotrophins (Seidah *et al.*, 1996). Pro-neurotrophins comprise 40-60% of the total neurotrophins secreted extracellularly (Heymach *et al.*, 1996; Mowla *et al.*, 1999; Farhadi *et al.*, 2000; Mowla *et al.*, 2001) and are capable of ligand signalling. They preferentially bind to the p75 receptor, have different biological effects compared to their mature form (Lee *et al.*, 2001a) and may depend upon presence of and binding to a co-receptor (sortilin) to mediate pro-apoptotic signalling (Nykjaer *et al.*, 2004).

1.15 Trk RECEPTORS

The Trk family of tyrosine kinase receptors was first described in 1986 as the product of the *trk* oncogene, a chimeric oncoprotein found in human colon carcinoma (Martin-Zanca *et al.*, 1986; Martin-Zanca *et al.*, 1989). The extracellular domains of Trk receptors consist of leucine-rich motifs utilised by other receptors as cell adhesion components. The intracellular domains of full-length Trk receptors consist of catalytic tyrosine kinase domains and are highly related, with approximately 80% amino acid

identity. TrkB and TrkC also exist as splice variants lacking the tyrosine kinase domain, which is replaced by a short cytoplasmic sequence.

Understanding the roles played by different trk receptors has been facilitated by the generation of mice carrying disrupted TrkA, TrkB and TrkC loci. The mutant mice exhibit severe neuronal deficiencies indicative of the key role that these signalling receptors play in the ontogeny of the mammalian nervous system. TrkA^{-/-} mice are small, display a wide array of sensory defects and have extensive neuronal cell loss in trigeminal, dorsal root and sympathetic ganglia (Smeyne *et al.*, 1994). Targeted disruption of the TrkB gene results in nervous system lesions with resultant deficiencies in trigeminal and nodose / peduncular ganglia (Klein *et al.*, 1993). TrkB targeted mice do not respond to stimuli, are unable to feed and as a consequence die in the neonatal period (Klein *et al.*, 1993). This, combined with the fact that these mice express truncated TrkB receptors, has challenged the study of effects of TrkB deletion. Generation of knock out mice lacking all TrkB receptor isoforms has been more revealing as mice lacking both full-length and truncated TrkB isoforms can survive up to 3 weeks after birth (Rohrer *et al.*, 1999). Unlike the targeted TrkA^{-/-} and TrkB^{-/-} mice, TrkC^{-/-} mice develop to birth and appear normal, respond to painful stimuli and take nourishment. However, after birth they display abnormal movements, suggesting a defect in proprioception. They have a limited lifespan (most die by P21) and most of their neuronal defects are in the dorsal root ganglia and in muscle afferents (Klein *et al.*, 1994).

1.16.1 Trk B

The TrkB family of transmembrane proteins serve as receptors for BDNF and NT4. Cloning and chromosomal localisation of the full-length human TrkB receptor was originally reported in 1995 (Nakagawara *et al.*, 1995). The human TrkB gene is unusually large and spans at least 590 kilobases (kb). It contains 24 exons. Using alternative promoters, splicing and polyadenylation sites, at least 100 isoforms can be created that can encode 10 proteins. Only 3 major isoforms are generated by the gene; the full-length receptor, a truncated isoform lacking the tyrosine kinase domain (TrkB-T) and an isoform lacking the tyrosine kinase domain but containing a Shc binding site (TrkB-T-Shc). TrkB-T-Shc is generated by the use of an alternative exon 19 and is expressed solely in brain (Stoilov *et al.*, 2002). As a general rule, full-length TrkB receptors are located in neuronal tissue and truncated TrkB receptors are distributed in non-neuronal tissues. BDNF binding to TrkB results in rapid degradation of TrkB receptors at the cell surface (Sommerfeld *et al.*, 2000). The sequences required for Trk internalisation have not been identified. In some central nervous system (CNS) neurons, TrkB receptors appear to be largely sequestered in intracellular vesicles and require a second signal, such as cAMP or Ca^{2+} , for efficient insertion of receptors into the surface membrane (Meyer-Franke *et al.*, 1998).

Truncated TrkB receptors without a kinase catalytic region were originally thought to lack a signalling role, their presence serving to act as a reservoir to bind and inactivate ligand (Biffo *et al.*, 1995) or to offer inhibitory receptor competition against full-length TrkB where they are co-expressed (Eide *et al.*, 1996; Ninkina *et al.*, 1996). Two forms

of the truncated TrkB receptor have been characterised, designated as TrkB.T1 and TrkB.T2 and containing intracellular domains of 23 and 21 amino acids respectively (Middlemas *et al.*, 1991). TrkB.T1 and TrkB.T2 have been shown to be capable of mediating BDNF-induced signal transduction (Baxter *et al.*, 1997; Rose *et al.*, 2003) and result in distinct modes of dendritic growth compared to full-length TrkB receptors (Yacoubian and Lo, 2000).

1.16.2 Trk B RECEPTOR SIGNAL TRANSDUCTION

Despite high conservation of their intracellular domains, including the catalytic tyrosine kinase (Atwal *et al.*, 2000), the Trk receptors can elicit different biological responses in the same population of neuronal cells (McAllister *et al.*, 1997; Bradbury *et al.*, 1998; Baldelli *et al.*, 2000). This is because distinct molecular mechanisms and signalling interactions are generated by each of the neurotrophins (Yamada *et al.*, 2002).

TrkB receptors are activated by interaction with NT4 or BDNF. Activation is a 2-step process and involves oligomerisation (dimerisation) of receptor molecules at the cell surface followed by autophosphorylation of their cytoplasmic tyrosine residues (Jing *et al.*, 1992). The resulting phosphotyrosines serve as anchors for binding downstream signalling elements and recruitment of Shc domain proteins, initiating further cascades of signalling events (Schlessinger and Ullrich, 1992). Three important features of Trk signalling are 1) activation of the PI3-kinase pathway and a subsequent block of apoptosis, resulting in cell survival and differentiation, 2) activation of Ras / Raf / MAPK/ERK kinase (MEK) / MAPK pathway, resulting in growth and differentiation

and 3) association with phospholipase C γ to regulate PKC activity and release of Ca²⁺ from intracellular stores (Bibel and Barde, 2000) (Figure 1.5).

Apoptosis regulates the number of CNS neurones and is important for the formation and organisation of brain structures. It is also a key feature in ovarian development and organisation (Coucovanis *et al.*, 1993; Pesce *et al.*, 1993; Ratts *et al.*, 1995; Reynaud and Driancourt, 2000). Neurotrophin activation of Trks and subsequent PI3-kinase activation prevents apoptosis, thus promoting cell survival (Wolter *et al.*, 1997; Gross *et al.*, 1998).

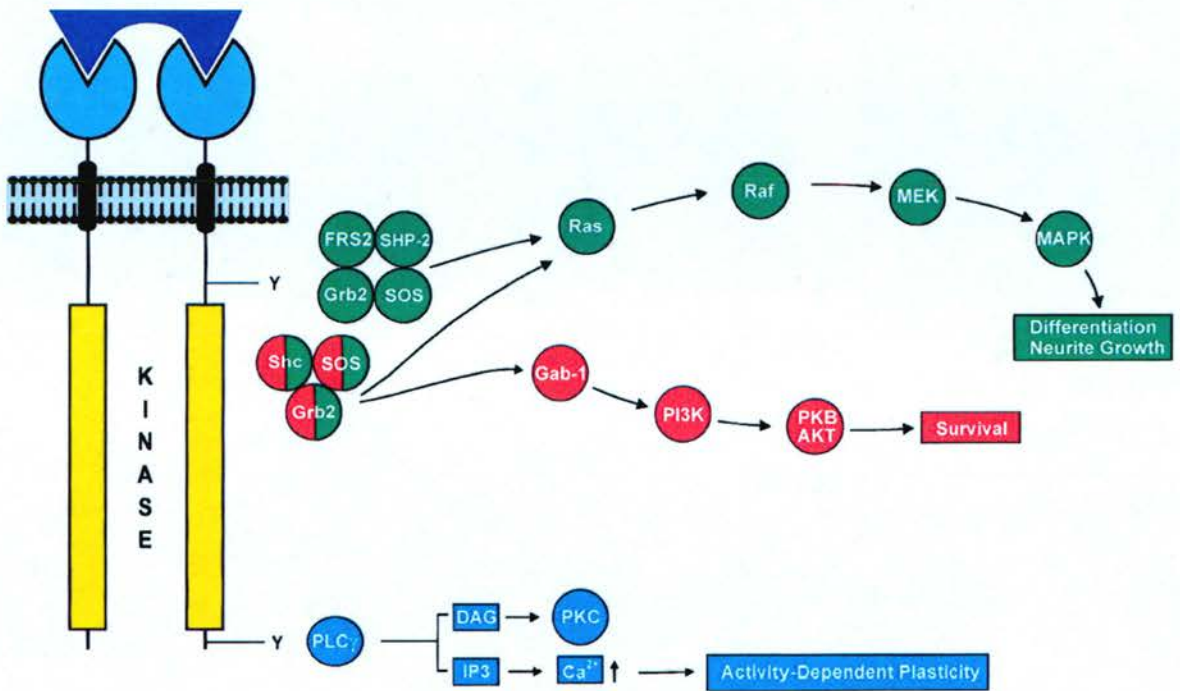


Figure 1.5 Diagram illustrating the main pathways signalled through the Trk receptors (Bibel and Barde, 2000).

1. Ras / Raf / MEK / MAPK pathway (green) induces differentiation of neurons and neurite growth. FRS2 tyrosine is phosphorylated in response to NGF and BDNF.
2. PKB / AKT pathway (red) phosphorylates Bad / inactivates Bcl2 and mediates survival by anti-apoptotic action.
3. PLC γ (blue) catalyses cleavage of the substrate PIP2 to DAG, which induces activation of PKC and IP3 leading to release of calcium from internal stores

1.16.3 p75 RECEPTOR

The p75 receptor is a member of the TNF receptor superfamily and can bind all neurotrophins with similar nanomolar affinity (Frade and Barde, 1998). The ability of p75 to bind NGF was originally detected in the 1970s, but the receptor was not fully described and cloned until ten years later (Chao *et al.*, 1986; Radeke *et al.*, 1987). The p75 receptor is a single transmembrane protein consisting of 425 amino acids with an intracellular carboxy-terminus and shares a similar intracytoplasmic domain structure (featuring a death domain with six α helices) to Fas and tumor necrosis factor receptor 1 (Myers *et al.*, 1994). The defining motifs of the TNF receptor family are cysteine repeats in the extracellular domain, where ligands bind. With the exception of the neurotrophins, all other known ligands of this receptor family are trimeric proteins that lead to trimerisation of the receptor following binding.

The p75 receptor is expressed widely in neurons of both the central and peripheral nervous system, and in a variety of nonneuronal cells, including ovary (Amano *et al.*, 1991; Wheeler and Bothwell, 1992; Dissen *et al.*, 1996; Anderson *et al.*, 2002b). Knockout mice lacking p75 show behavioural defects and decline in performance in Morris water maze testing, suggesting absence of p75 receptor signalling results in defects in cognitive function and/or global sensorimotor impairment. Stereological analysis of p75^{-/-} mice brains shows a 25% reduction in basal forebrain volume with associated loss of basal forebrain neurons, compared to wild-type mice (Peterson *et al.*,

1999). Complete ablation of the p75 receptor also results in defects of blood vessel formation (von Schack *et al.*, 2001).

Several different biological activities involve p75 receptor signalling, which requires multiple effector proteins. It is capable of, paradoxically, mediating cell death - either on ligand binding (Rabizadeh *et al.*, 1993; Rabizadeh and Bredesen, 1994), or on withdrawal of ligand (Barrett and Georgiou, 1996) - or of mediating cell survival. These effects are dependent not only on local expression of Trk receptors and ligand presentation but also on available downstream mediators (Rabizadeh and Bredesen, 2003). Cell cycle progression, axonal elongation (Yamashita *et al.*, 1999) and synaptic transmission (Yang *et al.*, 2002) also involve p75 receptor signalling.

The p75 receptor has the additional capacity to regulate binding affinity between Trks and their cognate ligands resulting in increased specificity of neurotrophin-receptor interactions (Benedetti *et al.*, 1993; Mahadeo *et al.*, 1994). Although p75 and Trk receptors do not bind to each other directly, there is some evidence that complexes are formed between the two receptors (Bibel *et al.*, 1999; Yano and Chao, 2000) and may interact to discriminate between neurotrophins. Indeed, BDNF, NT3 and NT4 can each bind to TrkB but in the presence of p75 only BDNF provides a functional response (Bibel *et al.*, 1999). BDNF triggers sympathetic neuron apoptosis by binding to p75 when TrkB is absent (Bamji *et al.*, 1998) and Agerman *et al* illustrated that BDNF and NT4 can elicit opposite outcomes in the same cell through interactions mediated by TrkB and p75 combined (Agerman *et al.*, 2000).

1.16.4 p75 RECEPTOR SIGNAL TRANSDUCTION

The p75 neurotrophin receptor is devoid of intrinsic catalytic activity and signal transduction necessitates the recruitment of cytoplasmic effectors. Interaction of neurotrophins with p75 can, therefore, result in activation of several different intracellular signalling pathways by utilising various effectors (Dobrowsky *et al.*, 1994; Dobrowsky *et al.*, 1995; Carter *et al.*, 1996; Bamji *et al.*, 1998; Yoon *et al.*, 1998; Bhakar *et al.*, 1999; Yamashita *et al.*, 1999). The nuclear factor- κ B (NF- κ B) pathway appears to promote survival (Ye *et al.*, 1999; Gentry *et al.*, 2000), whereas the jun N-terminal kinase (JNK)-p53-Bax pathway and sphingomyelin hydrolysis promote apoptosis. p75 signalling can also be regulated or modified by Trk receptor signalling (Dobrowsky *et al.*, 1995; Yoon *et al.*, 1998). Pro-apoptotic responses to the p75 receptor are largely suppressed in the presence of Trk signalling, and retention of p75 receptor activation of NF- κ B makes a synergistic contribution to survival (Maggirwar *et al.*, 1998; Hamanoue *et al.*, 1999).

The p75 receptor also binds non-neurotrophin ligands including the neurotoxic fragment of prion protein PrP-(106-126) (Della-Bianca *et al.*, 2001), the Ab peptide of the amyloid precursor protein, the predominant component of senile plaques in Alzheimer's disease (Yaar *et al.*, 1997; Kuner *et al.*, 1998; Perini *et al.*, 2002) and the rabies virus glycoprotein (Tuffereau *et al.*, 1998).

1.17 NEUROTROPHINS IN THE OVARY

Although originally discovered in the nervous system, expression of neurotrophins and their receptors has been described in a variety of non-neuronal tissues including the cardiovascular, immune, endocrine and reproductive systems. BDNF and TrkB have been identified in the adult avian ovary (Jensen and Johnson, 2001) and NT4 expression has also been localised to the oocyte in both rodent (Dissen *et al.*, 1995) and *Xenopus* (Ibanez *et al.*, 1992). NT4, and its receptors TrkB and p75 have been demonstrated in the human fetal ovary (Anderson *et al.*, 2002b), with expression of TrkB localised to the germ cells. The ovary, in fact, contains all neurotrophins NGF, BDNF, NT3 and NT4 (Ernfors *et al.*, 1990; Lara *et al.*, 1990; Berkemeier *et al.*, 1991; Hallbook *et al.*, 1991; Dissen *et al.*, 1995; Dissen *et al.*, 1996) and expresses all neurotrophin receptors TrkA, TrkB, TrkC and p75 (Klein *et al.*, 1989; Lamballe *et al.*, 1991; Ojeda *et al.*, 1992).

Before follicle formation is initiated, mRNA encoding neurotrophins and their respective receptors is present in the mammalian ovary (Dissen *et al.*, 1995; Anderson *et al.*, 2002b) and an increase in expression of genes encoding NT4 and TrkB, but not BDNF, has been found to occur with the initiation of folliculogenesis (Dissen *et al.*, 1995). Full-length TrkB receptors are expressed at low and seemingly unchanging levels in the oocytes and granulosa cells of both primordial and growing follicles. In contrast, truncated TrkB is selectively expressed in oocytes, where it is targeted to the cell membrane as primary follicles initiate growth (Paredes *et al.*, 2004). Knockout mice lacking all TrkB receptor isoforms, or those lacking both NT4 and BDNF, do not initially show follicular disruption, nor increase in ovarian cell apoptosis, indicating that

TrkB signalling may not be crucial to initial follicular assembly in the rodent. Absence of TrkB receptors does, however, result in a stage-selective deficiency in early follicular development that compromises the ability of follicles to grow beyond the primary stage, with decreased proliferation of granulosa cells and decreased FSH receptor expression (Paredes *et al.*, 2004). Ligand signalling via TrkB also appears to be central to oocyte survival during early follicular growth (Spears *et al.*, 2003; Paredes *et al.*, 2004). Knockout mice lacking the p75 gene appear to have normal numbers of ovarian follicles (unpublished data cited in (Dissen *et al.*, 1995)).

Mice carrying a null mutation of the NGF gene show deficient development of primordial follicles (Dissen *et al.*, 2001). NGF may be responsible for inducing expression of functional FSH receptors in newly formed follicles (Romero *et al.*, 2002), and its production increases proportionally with increase in follicle size (Mattioli *et al.*, 1999). TrkA and NGF are expressed in periovulatory follicles at the time of the preovulatory LH surge and are thought to play a role in processes leading to mammalian ovulation (Dissen *et al.*, 1996; Mayerhofer *et al.*, 1996). BDNF is normally present in follicular fluid (Seifer *et al.*, 2003) and a beneficial effect of BDNF on oocyte maturation in vitro has been reported (Seifer *et al.*, 2002a). Evidence for improved cytoplasmic maturation and developmental competence as well as nuclear maturation will be discussed in further detail in Chapter 5.

1.18 SUMMARY

In summary, this literature review has examined current knowledge pertinent to the subjects of germ cell survival, ovarian development and oocyte maturation.

Consideration has been given to activins, inhibins and neurotrophins, and their various receptors, as growth factors likely to be involved in the regulation of the development of the human fetal ovary as well as maturation of the bovine oocyte.

1.19 AIMS

The specific aims of this study were therefore to:

1. Investigate the expression and localisation of activin and the activin / inhibin receptors in the human fetal ovary, and to examine the effect of activin on germ cell proliferation / survival
2. Clarify expression of BDNF and NT4, and their receptors TrkB and p75, in the human fetal ovary and investigate their role in germ cell survival
3. Investigate the expression and localisation of BDNF and its receptors in the adult ovary and cumulus-oocyte complex and examine a potential role for BDNF in oocyte maturation, using a bovine model.

CHAPTER 2

MATERIALS AND METHODS

2.1 HUMAN FETAL TISSUE COLLECTION

Human fetal ovaries were obtained following medical termination of pregnancy.

Women gave consent according to national guidelines (Polkinghorne, 1989) and the study was approved by the Lothian Paediatrics / Reproductive Medicine Research Ethics Sub-Committee. Termination of pregnancy was induced by treatment with 200mg oral mifepristone followed 48 hours later by prostaglandin (Gemeprost; Beacon Pharmaceuticals, Tunbridge Wells, UK) 1mg 3 hourly per vaginum. None of the terminations were for reasons of fetal abnormality, and all fetuses appeared morphologically normal. Gestational age was determined by ultrasound examination prior to termination and confirmed by subsequent direct measurement of foot length.

Ovaries were dissected and either snap frozen and stored at -70°C , placed in 500 μl RNAlater (Ambion (Europe) Ltd, Huntingdon, Cambs, UK), fixed for immunohistochemical analysis, or further dissected and placed into culture. Fixation was carried out in Bouin's fluid for 5 hours, followed by transfer to 70% ethanol prior to processing into paraffin using standard methods.

2.2.1 ISOLATION OF RNA AND SYNTHESIS OF cDNA

Small amounts of RNA are present within tissues and must be amplified in order to identify and study gene expression. RNA is first converted to complimentary DNA (cDNA) using the enzyme reverse transcriptase. Polymerase chain reaction (PCR) can

then be performed on the cDNA using specific primers to manufacture copies of the required sequence.

Total RNA was isolated from fetal ovary (14 - 19 weeks) using either the RNeasy Mini Kit (Qiagen, Crawley, UK) for PCR, or TRIReagent for quantitative PCR analysis.

Using the RNeasy Mini Kit, tissue was homogenised and lysed in a volume of buffer proportional to the amount of tissue. The buffer contains guanidine isothiocyanate (GITC) and β -mercaptoethanol (β ME), which rapidly inactivates endogenous RNases.

This mixture was centrifuged and 70% ethanol then added to the supernatant to provide appropriate binding conditions. The mixture was agitated, then added to an RNeasy spin column and centrifuged at 12,000 G at room temperature (RT), allowing adsorption of the RNA onto the silica gel based membrane. The column was washed twice with washing buffer before eluting RNA with RNase-free sterile water. Alternatively, tissue was homogenised in 1ml TRIReagent, and allowed to settle prior to addition of chloroform, which separates the homogenate into aqueous and organic phases: RNA remains exclusively in the aqueous phase, DNA in the interphase and proteins in the organic phase. The aqueous phase was aspirated, transferred to a fresh collection tube and RNA then precipitated by addition of isopropanol. The pellet was washed with ethanol, briefly allowed to dry and then solubilised by adding RNase-free water.

RNA from both preparation methods was quantified using a spectrophotometer. RNA was then treated with DNase (Gibco, Paisley, UK) and reverse transcription performed using a first strand cDNA synthesis kit (Roche Diagnostics, Lewes, UK) Briefly, 3 μ g

DNase treated RNA was incubated with oligo (dT)₁₈ primer for 10 minutes at 65°C and then placed on ice. A reaction mix comprising buffer, reverse transcriptase, 100mM dithiothreitol (DTT), 10mM deoxynucleotide triphosphate (dNTP) and ribonuclease inhibitor was added to each tube to a final volume of 40µl. Tubes were incubated at 40°C for 2 hours and then cooled to 4°C. Resultant cDNA was then used for PCR amplification or stored at -20°C.

2.2.2 POLYMERASE CHAIN REACTION (PCR)

PCR is a method of *in vitro* nucleic acid synthesis, whereby a specific sequence of DNA is replicated. It is based on the DNA polymerisation reaction and requires two oligonucleotide primers, which are designed to lie either side of the DNA fragment to be amplified. These primers hybridise to opposite strands of the target sequence and are orientated so that DNA synthesis proceeds across the region between the primers. PCR involves repeated cycles of heat (95°C) to denature DNA, followed by temperatures to allow annealing of the primers to their complimentary sequences (50 - 60°C in the experiments described in the following chapters) and extension of the annealed primers with DNA polymerase (72°C). Since the extension products themselves are also capable of binding primers, successive cycles of amplification essentially double the amount of target DNA synthesised the previous cycle and result in an exponential accumulation of the specific target fragment.

PCR reactions were performed using 1µl cDNA samples, 5µM forward and reverse oligonucleotide primers and 2 x thermostart PCR mastermix (Abgene, Epsom, UK) in a total volume of 25µl. Use of thermostart DNA polymerase eliminates the possibility of non-specific priming during reagent preparation prior to PCR because the enzyme is inactive until high temperature incubation (95°C for 15 minutes). The mastermix format also offers benefits in terms of time taken for PCR preparation and reduces chances of preparation errors, therefore giving reliable and reproducible results. Specific primers were used, and these and the resultant product sizes are given in the relevant chapters. Primers were designed to span an intron to ensure that genomic DNA was not amplified. PCR cycling was carried out using MJ Research PTC-100 thermal cycler, for 35 cycles. The annealing temperature varied, depending on product and primers used. Control tubes were run in parallel for each PCR, one in which water replaced RNA and a second RT- sample in which reverse transcriptase was omitted, to ensure there was no genomic DNA contamination. After PCR, the size of product yielded was ascertained using electrophoresis. PCR products (10µl product + 2µl 6x loading buffer) and PCR marker (1Kb ladder; Invitrogen) were loaded on a 1.7% agarose gel containing ethidium bromide, placed in a tank containing 1 x Tris, boric acid, EDTA (TBE) buffer and electrophoresed at 90 – 110 V for 45 minutes. Products were visualised using ultraviolet light. The identity of all PCR products were confirmed by direct sequencing using an Applied Biosystems 373A automated sequencer.

2.2.3 LIGHTCYCLER QUANTITATIVE PCR

Quantitative PCR, also known as real-time PCR, enables either absolute or relative quantification of cDNA or RNA. It is based on the detection of a fluorescent reporter molecule (SYBR Green 1) that increases as a PCR product accumulates with each amplification cycle. SYBR Green 1 binds all double-stranded DNA and is monitored by measuring the increase in fluorescence throughout the cycle.

Quantitative PCR was performed using the Lightcycler system (Roche Molecular Biochemicals, Sussex, UK) to investigate changes in expression of the activin β A subunit over a range of gestations. Reverse transcribed RNA samples were diluted in water as indicated. 1 μ l diluted first-strand cDNA was added to a final volume of 10 μ l containing 2mM $MgCl_2$ and 0.5 μ M each of forward and reverse primer in 1 x LightCycler Fast Start DNA MasterSYBR Green 1 Master Mix (Roche Molecular Biochemicals). Signal acquisition was performed for each of 45 amplification cycles followed by continuous melt curve analysis to ensure product accuracy. Primers used are detailed in chapter 3.

Glyceraldehyde-3-phosphate dehydrogenase (GAPD) is an enzyme involved in glycolysis, and is important for multiple cell functions including membrane fusion, microtubule bundling, phosphotransferase activity, nuclear RNA export, DNA replication, DNA repair and apoptosis. It is constitutively expressed in almost all tissues at high levels. GAPD is used in quantitative PCR as a reference control for gene

expression between different ovary samples. Using a 16-week fetal ovary, a series of first-strand cDNA dilutions were made (1:10 to 1:100) and used to derive standard curves for GAPD and activin β A. The number of cycles needed to yield a fluorescent signal above background (the cross-over point, Cp) was plotted against the log of relative concentration using LightCycler software (Molecular Dynamics Ltd, Buckinghamshire, UK) yielding a straight line for each product. Quantification of ovarian β A mRNA expression was subsequently performed. For each experiment, both GAPD and activin β A amplification reactions were performed in duplicate for every cDNA sample used. Calculations for activin β A mRNA concentration were made relative to GAPD from the same sample to allow comparison between ovaries. Allowances for difference in amplification rate for GAPD and activin β A was achieved by determining the actual amount of amplification required to yield a signal for each target.

2.3.1 IMMUNOHISTOCHEMISTRY

The technique of immunohistochemistry involves localisation of an antigen (protein) within a section of tissue using a specific antibody, which is either labelled itself or detected by use of a secondary labelled antibody. A variety of different detection systems, including enzymatic reaction, radioactivity signal or fluorescent dyes, can be used to detect and visualise staining. A standard and widely used technique for immunohistochemical staining is the Avidin-Biotin Complex (ABC) method. Avidin is a large glycoprotein that has a high affinity for biotin and can be labelled with peroxidase.

Biotin is a vitamin B complex and can be conjugated to a variety of biological molecules including antibodies. The ABC method involves three stages: 1) application of unlabelled primary antibody, 2) application of biotinylated secondary antibody followed by 3) application of avidin-biotin-horseradish peroxidase (ABC-HRP) complex. The peroxidase is then developed by 3,3'-diaminobenzidine tetrahydrochloride (DAB) to produce a colorimetric end product.

False positive or non-specific staining may occur, and can be due to variety of reasons. Firstly, an antibody may bind non-specifically to other epitopes in a tissue or may be contaminated with other antibodies due to impure antigen used to immunize the host animal. This is particularly applicable to polyclonal antibodies. A good antibody is key, and this problem can be minimalised by careful selection. Secondly, tissues may contain endogenous peroxidase, which catalyses enzymatic detection and will produce DAB staining at the site of endogenous enzyme. To avoid this problem, tissues are exposed to dilute methanol prior to addition of primary antibody, saturating endogenous peroxidase and rendering it inactive. Endogenous biotin can also bind to primary antibodies. This is overcome by applying unconjugated avidin to the tissue, which binds endogenous biotin, and subsequent application of biotin to bind the avidin. Lastly, secondary antibodies may bind to epitopes in the original tissue as well as to the primary antibody. This is minimalised by applying the secondary antibody diluted in serum from the species that it was raised against.

Sections (5µm) were mounted on BDH Superfrost Plus slides (BDH Laboratory Supplies) and dried overnight at 50°C before processing for immunohistochemistry. Firstly, slides were dewaxed in xylene (5 minutes) and rehydrated by washes through ascending grades of ethanol (absolute ethanol, 95% industrial methylated spirits (IMS) and 70% IMS) to distilled water (20 second washes). Heat induced epitope retrieval (HIER) was used for some antibodies. Pre-treatment with citrate can break protein cross-links formed by formalin (Bouins) fixation and can uncover hidden antigenic sites. Where applicable, slides were immersed in boiling citrate (0.01M, pH 6.0) for 2 minutes and then cooled and washed with tap water. They were then washed in Tris-buffered saline (TBS; 0.05mol/l Tris, 0.85% NaCl, pH 7.6). All slides were incubated in 3% H₂O₂ in methanol for 30 min, to inhibit endogenous peroxidase activity. After rinsing in distilled water, slides were transferred into TBS for 5 min and blocked for 30 min in appropriate serum (Diagnostics Scotland, Carluke, UK) diluted 1:5 in TBS containing 5% bovine serum albumin. Sections were then blocked with avidin (0.01M; 15 min) and biotin (0.001M; 15 min) with 5 minute washes in TBS in between. Various primary antibodies were used, as described in relevant chapters. Optimal antibody concentrations were established in a series of preliminary experiments. Positive controls used tissue sections known to show positive staining for the antibodies tested, either human fetal testis (for activin/ inhibin subunits and receptors) or rat brain (for neurotrophins). Negative controls were used to confirm specificity of antibodies under investigation. In the case of monoclonal primary antibodies, the method substituted incubation with buffer in place of primary antibody, whilst primary antibody was replaced with non-

immune serum (from the same species) in the case of polyclonal antibodies under investigation. Negative control for BDNF experiments was created by pre-incubation of BDNF antibody with a specific BDNF blocking peptide (Santa Cruz, CA, USA). All were incubated in a humidified atmosphere at 4°C overnight. The following day, sections were washed and incubated for 30 min with a biotinylated secondary antibody at a dilution of 1:500 in the appropriate serum. Following washes in TBS, sections were incubated with ABC-HRP linked complex (Dako) according to the manufacturer's instructions. Bound antibody was visualised using DAB (Dako), which produces brown staining. Sections were counterstained with haematoxylin, dehydrated in graded ethanols and histoclear and then soaked in xylene. Slides were mounted with Pertex mounting medium and visualised by light microscopy. Images were captured using an Olympus Provis microscope (Olympus Optical Co., London) equipped with Kodak DCS330 camera (Eastman Kodak).

2.3.2 NUCLEAR DIAMETER MEASUREMENT

The size distribution of germ cells expressing activin β A subunit was determined in specimens of 18 – 19 weeks gestation. Sections (5 μ m) were mounted on BDH Superfrost Plus slides (BDH Laboratory Supplies) and dried overnight at 50°C before processing for immunohistochemistry as described above (section 2.5.1). Briefly, slides were incubated in 3% H₂O₂ in methanol for 30 min, to inhibit endogenous peroxidase activity. After rinsing in distilled water, slides were washed in TBS for 5 minutes and then blocked for 30 minutes in normal rabbit serum (Diagnostics Scotland, Carlisle, UK)

diluted 1:5 in TBS containing 5% bovine serum albumin. Sections were then blocked with avidin (0.01M; 15 min) and biotin (0.001M; 15 min) with 5 minute washes in TBS in between. Monoclonal primary antibody to the inhibin / activin β A subunit (E4), gift of NP Groome, was used at 1:1000 and incubated overnight at 4°C. Sections were then washed and incubated with rabbit anti-mouse biotinylated secondary antibody, at a dilution of 1:500. Following washes in TBS, sections were incubated with ABC-HRP linked complex (Dako) according to the manufacturer's instructions. Bound antibody was visualised using DAB (Dako), to produce brown staining. TBS was used in place of primary antibody for negative controls. Sections were counterstained with haematoxylin, dehydrated in graded ethanols and histoclear and then soaked in xylene and mounted with Pertex mounting medium.

Slides were visualised using the Provis image analysis system (Olympus Optical Co., London). To avoid bias, sequential non-overlapping frames were captured using the Provis image analysis system, and the diameter of all germ cell nuclei within the frame measured. Nuclear diameter, rather than germ cell diameter, was measured as this was easier to accurately define. Analysis in Professor Anderson's laboratory has, however, demonstrated a very close correlation between nuclear and germ cell diameter in the human fetal ovary (results unpublished, Ms N Fulton). Diameters of a total of 527 germ cell nuclei were measured. Strongly immunostained germ cells were classified as β A positive, all other germ cells as negative. For graphical representation, numbers of immunopositive and immunonegative cells were grouped in 1 μ m increments of nuclear

diameter. Data were analysed by the Mann-Whitney U test as the distribution deviated from normal. The Mann-Whitney U test is a non-parametric test used to assess whether two samples of observations come from the same distribution. The two samples must be independent, and observations ordinal or continuous. The null hypothesis assumes that two samples drawn from a single population will have equal probability distributions. The test involves the calculation of a statistic, U, whose distribution under the null hypothesis is known. In the case of larger samples, as in this case, there is a good approximation of U to a normal distribution.

2.3.3 HAEMATOXYLIN AND EOSIN STAIN (H & E STAIN)

H & E staining was utilised to check tissue morphology as well as to count germ cell number for time 0 samples relating to tissue culture experiments. Tissue sections were dewaxed in xylene for 5 minutes, rehydrated in graded alcohols (absolute ethanol 20 seconds, 95% IMS 20 seconds and 70% IMS 20 seconds) and rinsed in tap water prior to staining with Haematoxylin (5 minutes). Next, slides were rinsed in 1% acid alcohol (5 seconds), Scott's tap water (40 seconds) then Eosin (5 – 20 seconds), with rinses in tap water between each stage. Finally, slides were dehydrated in graded alcohols (70% IMS 20 seconds, 95% IMS 20 seconds, absolute alcohol 20 seconds x 2) then soaked in HistoClear (5 minutes) followed by xylene (5 minutes) before mounting in Pertex.

2.4 TISSUE CULTURE

Human fetal ovary tissue was dissected and cultured in α -minimal essential medium (α -MEM) with phenol red (GibcoBRL, Life Technologies). This medium has been shown to be most effective at supporting human ovarian tissue in culture (Wright *et al.*, 1999), possibly because it contains amino acids, vitamins, ribonucleosides and deoxyribonucleosides that are not present in simple salt solutions. α -MEM was supplemented with 2mM pyruvate, 2mM glutamine, 1 x insulin / transferrin / selenium (ITS) supplement and 3mg/ml BSA, and with Penicillin, Streptomycin and Amphotericin. Serum albumin and ITS have been shown to be effective replacements for serum in culture (Wright *et al.*, 1999), and allow testing of growth factor effects in a serum-free environment.

Fetal ovaries were dissected in pre-warmed medium. Fetal ovaries were initially halved and one half removed as 'time 0' uncultured control tissue before further dissection to yield tissue fragments approximately 1mm³. 'Time 0' tissue was immediately washed in phosphate buffered saline (PBS) and fixed in Bouin's fluid for 1 hour, then transferred to 70% ethanol followed by processing into paraffin using standard methods. Tissue fragments were cultured in 400 μ l medium, enough to just cover but not submerge them. 4 – 6 fragments were transferred to designated wells containing inserts (Millicell-CM; Millipore UK Ltd, Watford, UK) in a 24 well tissue culture plate. Further wells were partially filled with medium to maintain humidity in the culture plate.

A total of 7 fetal ovaries were used in the first set of culture experiments (chapter 3). Tissue was cultured for 17 hours in a humidified incubator at 37°C and 5% CO₂. For each experiment, two treatment groups were cultured with 100ng/ml recombinant human activin A (R&D systems, Abingdon, UK). Two further groups acted as controls. Bromodeoxyuridine (BrdU) was supplemented to culture media at 10µl/ml as a marker of cell proliferation. In each experiment BrdU was present in the media of one control and one treatment group for the duration of the experiment. BrdU was added to the other control and treatment group for the final 4 hours of tissue culture only. Analysis of germ cell number in these parallel cultures confirmed that changing the medium 4 hours before the end of the culture period did not influence the results.

In a second set of experiments, a further 5 fetal ovaries were used (chapter 4). Tissue was cultured for 24 hours in a humidified incubator at 37°C and 5% CO₂. As a modification of the method used previously, ovaries were completely dissected into tissue fragments measuring approximately 1mm³. In each experiment, 4 – 6 tissue fragments were randomly selected as ‘time 0’ uncultured control tissue, immediately washed in PBS and fixed in Bouin’s fluid for 1 hour before transferring to 70% ethanol and processing into paraffin using standard methods. Further fragments were then transferred to designated wells containing inserts in a 24 well tissue culture plate. Six groups were cultured in total, five different treatment groups and one control group. Treatment groups were supplemented with either 100ng/ml NT4 (Peprotech EC Ltd), 10µg/ml anti-NT4, 100ng/ml NT4 and 10µg/ml anti-NT4, 100ng/ml BDNF (Peprotech

EC Ltd) or 100ng/ml BDNF and 10µg/ml anti-NT4. Additionally, 10µl/ml BrdU was used in all groups as an indicator of cell proliferation. No media exchange or supplementation was performed during these experiments.

At the end of culture, tissue fragment groups were washed in PBS, fixed in Bouin's fluid for 1 hour, transferred to 70% ethanol followed by processing into paraffin using standard methods.

2.5 QUANTIFICATION AND ANALYSIS OF CULTURED TISSUE

Serial sections (5µm), 4 sections per slide, were mounted on BDH Superfrost Plus slides (BDH Laboratory Supplies) and dried overnight at 50°C. Mounting 4 tissue sections per slide reduced the number of slides required, and only every 4th slide was processed for immunohistochemistry (as per section 2.5.1). Formalin grade α-BrdU monoclonal antibody (Roche, UK) was used at a dilution of 1:30 and incubated at 4°C overnight. On completion of the immunohistochemistry protocol sections were counterstained with haematoxylin (2 minutes), dehydrated and mounted.

Sections of uncultured 'time 0' and cultured tissue were analysed to investigate the effect of culture and of supplemented growth factors on the number and proliferation of germ cells and non-germ cells. Analysis of every 4th slide equated to analysis of tissue at intervals of over 80 µm, ensuring that the same cells could not be counted more than once. Analysis was carried out blind using the Area Fraction Probe in the Stereologer

software programme (Systems Planning and Analysis Inc, Alexandria, VA, USA) and using an Olympus BH-2 microscope (Olympus UK Ltd., London, UK) fitted with a Prior automatic stage (Prior Scientific Instruments Ltd., Cambridge, UK). This programme randomly selects frames within an outlined tissue fragment area, and thus minimises observer bias. In the first set of experiments, counting was performed using a 121-point grid in the eyepiece of the microscope. Cells lying directly beneath the intersection on the grid were identified as germ cell or non-germ cell and recorded. Cell classification as germ cell was made on the basis of histological appearance – larger cell, larger nucleus, visible chromatin, located within germ cell nest – or non-germ cell (pre-granulosa / endothelial cell / blood cell). Numbers proliferating (ie. immunostained for BrdU) for each cell type was also recorded. The number of points lying outside the tissue in any grid was also recorded and the total cell numbers were corrected for this (using the formula: cells counted x 121 / points on tissue). Areas selected by the randomised stage with less than 50 % grid points overlying tissue were not analysed. Average number of germ cells per grid was calculated for each experimental condition.

In the second set of experiments, stereology software had been up-graded, and analysis was performed using Image-Pro Plus 4.5.1 with Stereologer-Pro 5.1 plug-in software (Media Cybernetics UK, Wokingham, Berkshire, UK). As previously described, tissue areas were identified by outline and subsequently analysed following randomised selection. The area to be analysed was displayed on a computer screen (instead of visualising down the microscope), which was overlaid by a 63-point grid. Cells lying directly beneath the intersection on the grid were counted and classified as germ cell /

non-germ cell, as well as documenting presence or absence of BrdU staining. The number of points lying outside the tissue in any grid was also recorded and total cell numbers corrected for this (using the formula: cells counted \times 63 / points on tissue). Areas selected by the randomised stage with less than 50 % grid points overlying tissue were not analysed.

Data was imported from stereology results recording to excel spreadsheets for statistical analysis. Each repetition of the experiment was initially analysed separately to derive mean number of germ cells and non-germ cells for time 0 tissue, as well as number of stained germ cells, unstained germ cells, stained non-germ cells and unstained non-germ cells for culture control and treatment groups. Results were then averaged across repeat experiments, calculating mean results (and standard error of the mean, SEM) for cell types (stained or unstained) for time 0, control and culture treatment groups.

In the activin culture experiments, statistical analysis comparing control and treatment groups was performed using Student's t-test. In the neurotrophin culture experiments, results were analysed using analysis of variance (ANOVA) with post hoc Bonferroni correction. The t-test could have been used to test differences between means of the control and different treatment groups, but would have led to inflation of type I error rate (because the likelihood of finding something by chance is increased by making multiple comparisons). ANOVA was therefore used to test for significant differences among the means of control and treatment groups. This has the advantage of avoiding increasing the

type I error rate, but only identifies a significant difference between groups, not which groups are significantly different from each other. Post-hoc comparison was then used to find which groups were significantly different from each other and which were not.

2.6 BOVINE OVARY COLLECTION

Bovine ovaries were obtained from Galashiels abattoir. Ovaries were recovered from the animals, stored at 37°C after collection and transported to the laboratory in a thermos flask with pre-warmed heat packs. Ovaries were then washed in warm PBS and either snap frozen and stored at -70°C, fixed for immunohistochemical analysis or follicles aspirated to yield immature cumulus-oocyte complexes (COCs) for maturation culture.

Fixation of bovine ovaries was carried out in Bouin's fluid overnight, followed by transfer to 70% ethanol prior to processing into paraffin using standard methods. To facilitate their handling prior to fixation, COCs were first embedded in 50µl droplets of collagen solution, using a method described by Izadyar *et al* (Izadyar *et al.*, 1998).

Collagen solution was prepared by dissolving 4.2mg type I rat-tail collagen in 1ml 0.1M acetic acid (BDH) and mixing with an equal volume of 2x tissue culture medium 199 (TCM 199), pH 7.2 immediately before use. Droplets containing COCs were incubated at 37°C for 10 minutes to set, then fixed in Bouin's fluid for one hour and transferred to 70% ethanol prior to processing into paraffin using standard methods.

2.7 BOVINE CUMULUS-OOCYTE COMPLEX (COC) COLLECTION

COCs were aspirated from 4 – 8mm antral follicles visible on the surface of the ovary using a syringe and 18G needle. Studies suggest that oocytes obtained from follicles less than 3mm diameter have significantly lower rates of maturation and development to blastocyst stage (Pavlok *et al.*, 1992; Lonergan *et al.*, 1994b; Blondin and Sirard, 1995).

All ovaries obtained were aspirated, despite presence or absence of corpora lutea. Cows in follicular or luteal phases of the ovulatory cycle, or even pregnant at the time they are killed, do not show any marked difference in the maturational competence of oocytes (Trounson *et al.*, 2001). Follicular aspirate was maintained at 38°C and allowed to settle. The cellular debris was then transferred to a grid-marked 100mm petri-dish containing 5ml 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-buffered TCM 199 with 10% (heat inactivated) fetal calf serum (FCS) to allow sorting and selection of COCs. All procedures were carried out in a laminar flow hood using a Leica microscope with heated stage at 38°C.

2.8.1 ISOLATION OF RNA AND SYNTHESIS OF cDNA

RNA was extracted from whole bovine ovary, quartered for ease of handling, using the RNeasy mini kit (Qiagen, Crawley, UK) as previously described (section 2.4.1).

RNA was extracted from bovine COCs, cumulus and single oocytes as per method described by Young *et al* (Young *et al.*, 1998), using the QIAshredder cell lysate homogeniser (Qiagen, Crawley, UK) followed by the RNeasy mini kit (Qiagen, Crawley, UK). COCs, cumulus or single oocytes were vortexed in 350µl buffer containing GITC and βME to inactivate endogenous RNases. Buffer was also added to an empty tube, designated non-embryo control (NEC), which was used to control for reagent contamination. Samples, including NEC, were then homogenised by adding to a QIA shredder column and centrifuging for 2 minutes at 13,000rpm. An equal volume of

70% ethanol was added to the homogenised lysate and mixed well. Samples were applied to RNeasy mini spin columns and centrifuged for 30 seconds at 10,000rpm. Columns were washed twice with wash buffer as per manufacturer's instructions and RNA was eluted with 50µl RNase free water. RNA precipitation was performed using Pellet Paint (Novagen). Pellet Paint is a visible dye-labelled reagent formulated specifically for use in alcohol precipitation of nucleic acids. Once RNA is precipitated from solution, the pink pellet is easily located and prevents loss during handling. Pellet Paint (2µl) was added to each tube of RNA and mixed with 5µl (0.1 volume) 3M sodium acetate. Thereafter 100µl (2 volumes) 100% ice cold ethanol was added to each tube and vortexed briefly. Samples were incubated for 2 minutes at room temperature, and subsequently centrifuged at 14,000rpm for 30 minutes at 4°C. The supernatant was removed and the pellet rinsed with 500µl ice cold 70% ethanol, which was vortexed briefly and then centrifuged at 14,000rpm for 20 minutes at 4°C. The supernatant was aspirated, the tube spun again briefly and any further supernatant aspirated to remove as much ethanol as possible. Pellets were air-dried on ice for approximately 10 – 15 minutes. Each pellet was then re-suspended in RNase free water, and transferred to a PCR tube for reverse transcription.

RNA was heated to 65°C for 10 minutes to denature, then chilled on ice. Reverse transcription was performed using a bulk first strand cDNA synthesis kit (Amersham, UK), whereby 5µl bulk first-strand reaction mix was mixed with 1µl random hexamer primer (pd(N)₆) and 1µl DTT and added to the denatured RNA. The mixture was

incubated at 37°C for 1 hour and cooled to 4°C thereafter. A control tube in which reverse transcriptase was omitted, designated RT-, was also included to ensure there was no genomic DNA contamination. Resultant cDNA was either used immediately for PCR amplification, or stored overnight at -20°C.

2.8.2 SINGLE CELL PCR

PCR used 2µl cDNA samples and 2 x thermostart PCR mastermix (Abgene, UK). Specific primers were used, at a concentration of 250nM, and these and the resultant product sizes are given in chapter 5. PCR cycling was carried out using a Hybaid Omnigene thermal cycler, for 40 cycles. The annealing temperature varied, depending on product and primers used. Control tubes were run for each PCR to ensure there was no genomic DNA contamination, one in which water replaced cDNA, and a second RT-sample in which reverse transcriptase was omitted. Reagent blank control tube for RNA extraction (NEC) was also included. After PCR, the size of product yielded was ascertained using electrophoresis. PCR products (10µl product + 2µl 6x loading buffer) and PCR marker (1Kb ladder; Invitrogen) were loaded on a 1.7% agarose gel containing ethidium bromide (0.5µg/ml), placed in a tank containing 1 x TBE buffer and run at 90 – 110 V for 45 minutes. Products were visualised using ultraviolet light. The identity of all PCR products was confirmed by direct sequencing.

2.9 IMMUNOHISTOCHEMISTRY

Sections of bovine ovary or collagen-embedded COCs (5µm) were mounted on BDH Superfrost Plus slides (BDH Laboratory Supplies) and dried overnight at 50°C before processing for immunohistochemistry as previously described (section 2.5.1). Briefly, slides were incubated in 3% H₂O₂ in methanol for 30 min to inhibit endogenous peroxidase activity. After rinsing in distilled water, slides were washed twice in TBS for 5 min and blocked for 30 min in appropriate serum (Diagnostics Scotland, Carlisle, UK) diluted 1:5 in TBS containing 5% bovine serum albumin. Sections were then blocked with avidin and biotin (both from Vector, Peterborough, UK) with washes in TBS in between. The following primary antibodies were used: BDNF (N20) (Santa Cruz, CA, USA), which was used at 1:100; TrkB (chicken polyclonal, Promega, Southampton, UK) used at 1:25; Truncated trk B (Santa Cruz, CA, USA) used at 1:50; p75 (Neomarkers, CA, USA) used at 1:25. All were incubated at 4°C overnight. Sections were then washed and incubated for 30 min with biotinylated secondary antibody as appropriate, at a dilution of 1:500. Following washes in TBS, sections were incubated with avidin-biotin-horseradish peroxidase linked complex (Dako) according to the manufacturer's instructions. Bound antibody was visualised using 3,3'-diaminobenzidine tetrahydrochloride (Dako). Pre-absorbed BDNF blocking peptide (Santa Cruz, CA, USA) or non-immune serum was used in place of primary antibody for negative controls.

Sections were counterstained with haematoxylin, dehydrated, mounted and visualised by light microscopy. Images were captured using an Olympus Provis microscope (Olympus Optical Co., London) equipped with Kodak DCS330 camera (Eastman Kodak).

2.10 *IN VITRO* MATURATION

Maturation medium used was a modification of a recipe described by Moor *et al* in 1977 originally used to successfully mature sheep oocytes (Moor and Trounson, 1977) and based on tissue culture medium 199 (TCM 199), a medium that has been demonstrated to contain components necessary for optimal bovine oocyte maturation (Lonergan *et al.*, 1994a; Gandhi *et al.*, 2000).

COCs were transferred through three wash plates containing 1.5ml HEPES-buffered TCM 199 with 10% fetal calf serum, before transfer in minimal volume to 1.5ml maturation wash comprising serum-free bicarbonate-buffered TCM 199 supplemented with 0.01iU/ml FSH (Ovagen), 0.125iU/ml LH and 2µg/ml β-estradiol. COCs were then randomly selected into groups and transferred into 500µl maturation medium in 4-well Nunc plates. There were six different maturation treatment groups: medium was either 1) serum-free or supplemented with 2) 10% fetal calf serum, 3) 100ng/ml recombinant human BDNF (PeproTech EC Ltd), 4) 10ng/ml recombinant human BDNF, 5) 5 µg/ml monoclonal α-human BDNF or 6) both 10ng/ml recombinant human BDNF and 5µg/ml monoclonal α-human BDNF.

COCs were cultured under mineral oil at 38.5°C in a humidified incubator with 5% CO₂. After 26 hours maturation culture COCs were stripped and either processed for immunocytochemistry to assess meiotic progression or activated to produce parthogenic embryos. Cumulus was stripped by mechanical vortex (repeated aspiration using a Gilson pipette) and incubation in 300iU/ml hyaluronidase in serum-free HEPES buffered Synthetic Oviduct Fluid (HSOF) consisting of 108 mM NaCl, 7.2 mM KCl, 1.2 mM KH₂PO₄, 5 mM NaHCO₃, 20 mM HEPES, 0.33 mM Na-Pyruvate, 1.7 mM CaCl₂, 0.5 mM MgCl₂, 3.3 mM Na-Lactate, 1.5 mM glucose, 3 mg/ml BSA (fatty acid free), pH7.4, osmolarity 265-275 (Thompson *et al.*, 1995) for 60 – 90 seconds. Enzymatic action was neutralised by transferring oocytes through subsequent washes of HSOF supplemented with 10% FCS.

In a second group of experiments, oocytes were stripped of cumulus prior to maturation. Only completely denuded oocytes were selected and washed in serum-free base maturation medium (see above). Five cumulus-free maturation treatments were evaluated consisting of serum-free medium (negative control), or medium supplemented with 10% FCS (positive control), 10 ng/ml of recombinant human BDNF, 5 µg/ml monoclonal anti-human BDNF or both 10ng/ml recombinant human BDNF and 5 µg/ml anti-human BDNF. In each experimental replicate a sixth group of cumulus enclosed oocytes were matured in base maturation media supplemented with 10% FCS (cumulus positive control). As previously, oocytes were cultured at 38.5°C in a humidified incubator with 5% CO₂ for 26 hours and subsequently either processed for

immunocytochemistry to assess meiotic progression or activated to produce parthenogenetic embryos.

2.11 IMMUNOCYTOCHEMISTRY

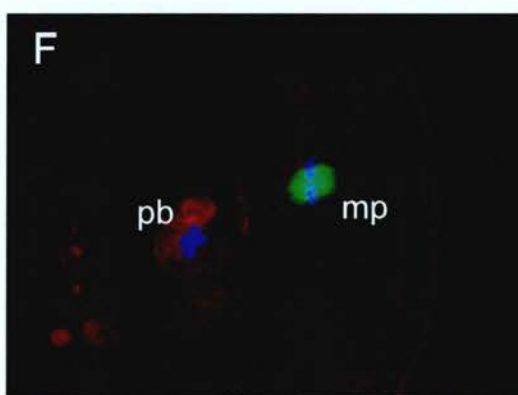
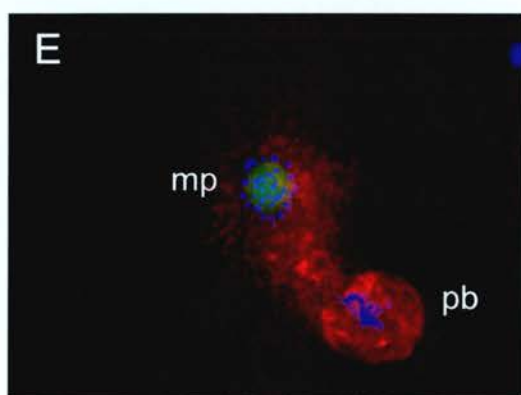
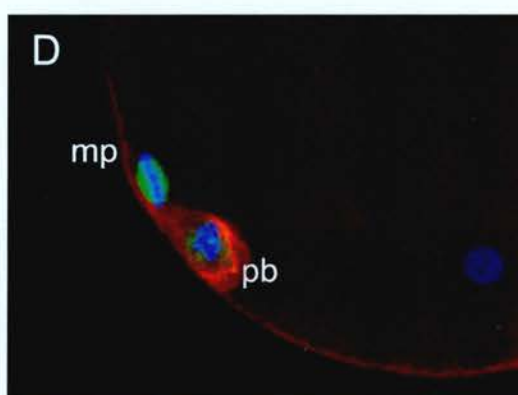
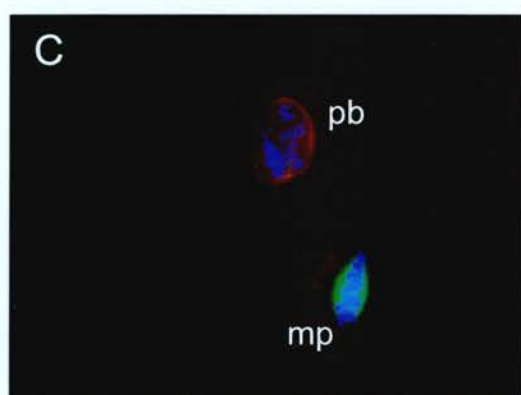
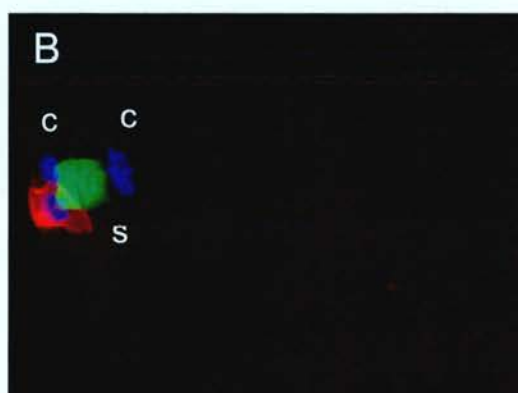
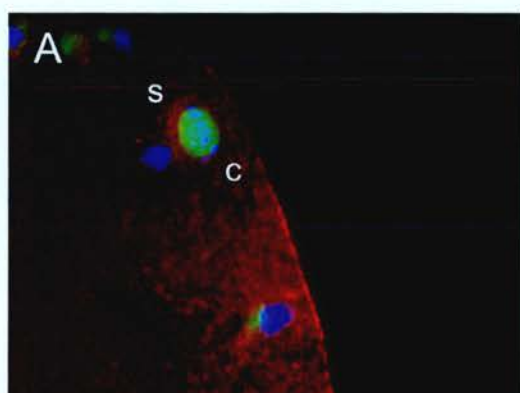
Stripped oocytes were fixed and immunostained for microtubules and microfilaments using a modification of the method described by Messinger and Albertini (Messinger and Albertini, 1991). Maturation to MII was assessed by immunocytochemical staining to visualise spindle morphology, condensed chromatin and segregation of the first polar body. Microtubule stabilising buffer comprising 5xSB (0.1M Pipes, 5mM MgCl₂, 2.5mM ethylene glycol bis (β aminoethyl ether)-NNN'N'-tetra acetic acid (EGTA), pH6.9 in NaOH), 1M DTT, deuterium oxide (Aldrich) and distilled water was incubated with Triton X100 (BDH) and 37% formaldehyde to make Complex Fix. Triton X-100 is a detergent that is used for improving antibody penetration. Stripped oocytes were incubated in Complex Fix for 30 minutes at 37°C and then washed 3 times in 0.1% goat serum before blocking in 10% goat serum for 1 hour at room temperature. Each maturation group of oocytes was processed individually. Oocyte groups were then incubated in the dark at 37°C for 1 hour in 50μl droplets of 5% goat serum with rhodamine phalloidin and α-tubulin FITC, washed 3 times in 10% goat serum and then mounted in Vectashield and 4'6-diamidino-2-phenylindole (DAPI; Vector, Peterborough, UK). Oocytes were partially squashed with a coverslip that was then sealed using clear nail varnish.

2.12 ANALYSIS OF IMMUNOCYTOCHEMISTRY

Slides were visualised using fluorescent microscopy and each oocyte scored for meiotic progression (Figure 2.12.1). Rhodamine phalloidin binds and stabilises microfilaments and is seen as red under fluorescence. FITC (fluorescein) is a small organic molecule conjugated (in this case) to α -tubulin monoclonal antibody in order to visualise spindle filaments. It is seen as green under fluorescence. DAPI is a fluorescent stain that binds strongly to double-stranded DNA and is excited with ultraviolet (UV) light (blue emission).

Figure 2.12.1 Oocyte immunocytochemistry demonstrating microfilaments (red), microtubules (green) and chromatin (blue). **A**, metaphase I. Chromosomes aligned along spindle. **B**, anaphase I. Homologous chromosomes separated to opposites poles of spindle. **C – F**, metaphase II. Chromatin aligned at metaphase plate by spindle fibres. Polar body containing chromatin also seen

s spindle, **c** chromosomes, **mp** metaphase plate, **pb** polar body



2.13 PARTHENOGENETIC ACTIVATION

Stripped oocytes were activated for 5 minutes at room temperature with 5 μ M ionomycin in HSOF with 10% FCS. Ionomycin is a calcium ionophore used to increase the level of intracellular Ca²⁺. Stripped oocytes were subsequently washed twice in HSOF with 10% FCS and then washed in SOFaaBSA (HEPES-free SOF supplemented with 1mM L-Glutamine, 8 mg/ml fatty acid free bovine serum albumin, 1 x essential amino acids, 1x nonessential amino acids, pH7.4, osmolarity 265-275) (Thompson *et al.*, 1995; Walker *et al.*, 1996). Oocytes were then incubated for 4 hours in SOFaaBSA containing 2.5mM DMAP and 10 μ g/ml cycloheximide at 38.5°C in humidified low O₂ and 5% CO₂ conditions. DMAP is a serine-threonine protein kinase inhibitor that blocks germinal vesicle breakdown and cell cycle progression in immature oocytes, and reduces phosphorylation of cell cycle regulatory proteins including MPF and CSF. Cycloheximide is an inhibitor of protein biosynthesis. Following activation, oocytes were washed three times in SOFaaBSA and finally transferred into 4-well Nunc plates for culture in 500 μ l SOFaaBSA under mineral oil. Embryos were examined for cleavage at 24 hours post activation (day1), had 10% FCS supplemented to media on day 5 of culture and were assessed for blastocyst development on day 7.

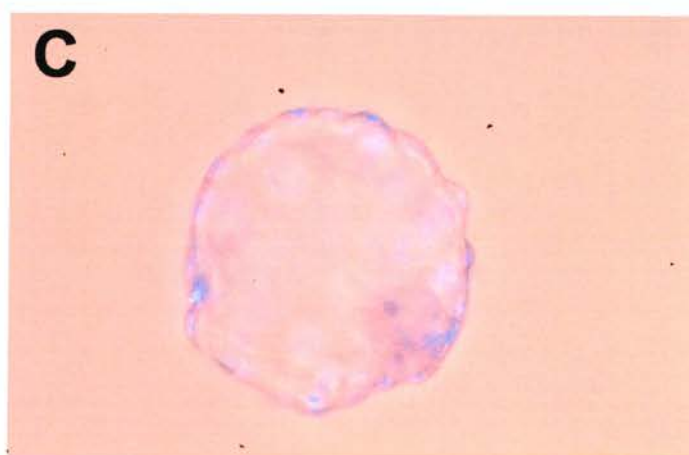
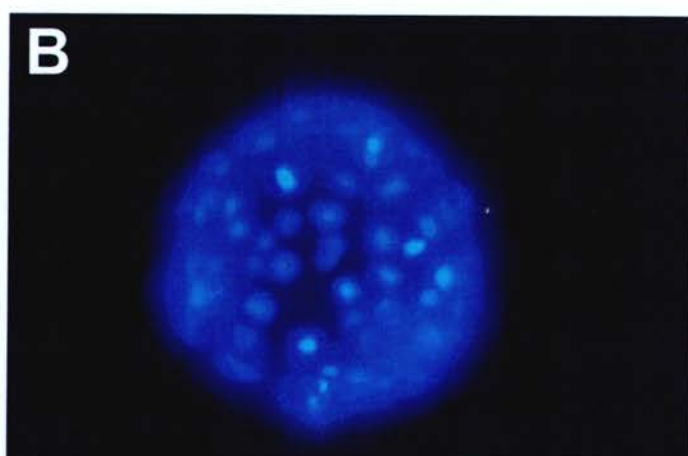
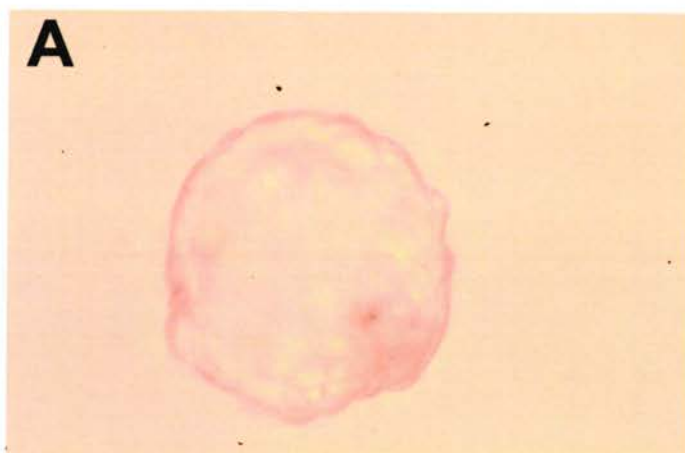
2.14 QUANTIFICATION AND ANALYSIS OF BLASTOCYST FORMATION

Blastocysts were stained by incubation with 10 μ g/ml Hoechst 33342 (bisbenzimidazole) for 15 minutes. Hoechst 33342 binds to nuclear DNA with high specificity and photostability, and is seen as blue under UV wavelengths. Blastocysts were then

mounted on glass slides and nuclear number assessed by counting under fluorescent microscopy (Figure 2.14).

Figure 2.14 Analysis of blastocyst nuclear number.

Blastocyst seen under light microscopy (A). Blastocyst visualised under fluorescent microscopy following staining with Hoechst (B). Note: nuclear counts were performed whilst the blastocyst was intact. Minor alterations in microscope focus enable all stained nuclei to be counted. The blastocyst was subsequently squashed by application of a cover slip, and nuclear counts repeated to confirm results. Superimposed image of blastocyst under light microscopy with fluorescence (C).



CHAPTER 3

EXPRESSION AND DISTRIBUTION OF

INHIBIN / ACTIVIN SUBUNITS AND RECEPTORS

AND EFFECT ON GERM CELL SURVIVAL AND

PROLIFERATION

3.1 INTRODUCTION

Germ cell survival is key to the reproductive lifespan of the ovary and is determined by the continuing presence of oocytes within primordial follicles. These are formed during fetal life in the human. The number of follicles formed is dependent upon the balance between early germ cell proliferation and loss, as well as the interaction between oocyte and somatic cells (Hirshfield, 1991; McLaren, 1991). The pathways regulating this crucial process are largely unknown, although a number of genes necessary for the formation of a normal complement of primordial follicles have been identified (Matzuk, 2000; Amleh and Dean, 2002). During the period leading up to primordial follicle formation there is massive oogonial mitotic proliferation, both during and after migration from the yolk sac to the nephrogonadoblastic ridge. The oogonia associate with somatic cells resulting in syncytial nests of germ cells intermingled with pregranulosa cells and surrounded by a meshwork of ovarian stroma (Byskov, 1986; Pepling and Spradling, 2001; Sawyer *et al.*, 2002). The germ cells subsequently enter meiosis only to arrest at diplotene of the first meiotic division. By mid-gestation, the human fetal ovary contains its maximal number of germ cells. This peak is followed by widespread germ cell loss, by apoptosis, (De Pol *et al.*, 1997; Vaskivuo *et al.*, 2001) so that less than 20% survive by the time of birth (Baker, 1963). Primordial follicles are formed as oocytes become individually surrounded by somatic cells (Hirshfield, 1991) and it is this interaction that has been identified as the event that determines oocyte survival and presumably protection against apoptosis. A number of locally derived growth factors have been identified to be crucial to germ cell survival at early developmental stages, such as BMP4 (Fujiwara *et al.*, 2001a) and kit ligand (Manova *et*

al., 1990; Godin *et al.*, 1991), and following primordial follicle formation, such as GDF9 (Dong *et al.*, 1996). However, little information is available regarding factors regulating oogonial proliferation and primordial follicle formation, particularly in the human.

Activins and inhibins are members of the TGF β family. The component α and β subunits can be combined into biologically active $\alpha\beta$ inhibin or $\beta\beta$ activin dimers with generally opposing functional effects, and are produced in both gonad and pituitary (Burger and Igarashi, 1988; Chen, 1993; Mather *et al.*, 1997). Members of the TGF β superfamily can influence many facets of cell lineage and activity including proliferation, differentiation, adhesion, motility and apoptosis. Many are involved in embryonic development as well as adult tissue homeostasis (Mishina *et al.*, 1999; Padgett and Patterson, 2001). The activins, together with other members of the TGF β superfamily, signal through membrane-bound serine-threonine kinase receptors. They bind to a type II receptor (ActRIIA or ActRIIB), which recruits and phosphorylates a type I receptor with subsequent modulation of gene expression via Smad protein activation (Massagué, 1998). Several type I receptors, termed activin-like kinases (ALKs) have been identified. ALK4 (ActRIB) and ALK2 (ActRIA) are believed to be the preferential activin receptors. Whilst the Smad proteins are the central elements in the activin receptor signalling pathway, other signalling pathways may mediate at least some of the diverse biological responses of the TGF β superfamily, including MAPK pathways (Massagué, 2000; Mulder, 2000). The initial steps coupling the activin type II serine-threonine kinase receptor to MAPK are yet to be defined, although signalling via

TGF β -activated kinase 1 (TAK1) results in activation of stress-activated p38 and activating transcription factor 2 (ATF2), which can interact with Smad3 and Smad4 to mediate transcription (Cocolakis *et al.*, 2001; Derynck *et al.*, 2001).

A substantial body of evidence has accrued that activins and inhibins are regulatory factors in the adult ovary involved in a wide range of functions, including folliculogenesis, granulosa and theca cell proliferation, and steroidogenesis, (Mizunuma *et al.*, 1999; Knight and Glister, 2001; Findlay *et al.*, 2002) as well as oocyte maturation (Alak *et al.*, 1998; Izadyar *et al.*, 1998). Activin is expressed in the human gonad at very early stages of development (Harkness and Baird, 1997) and there are limited data indicating the expression of activin and inhibin in the developing human ovary following primordial follicle formation (Rabinovici *et al.*, 1991; Harkness and Baird, 1997). However no data are available regarding the presence of activin and its cognate receptors prior to primordial follicle formation in the human ovary. The expression and distribution of inhibin/activin subunits in the human fetal ovary during the period of oogonial proliferation leading up to primordial follicle formation were therefore explored. Location of activin receptors, as well as the inhibin receptor betaglycan (Lewis *et al.*, 2000), were also investigated to identify potential sites of action of these regulatory factors during this period of development. As oogonia in the mid-trimester human fetal ovary were found to express activin subunits and receptors, the effect of activin A on germ cell survival and proliferation was investigated using an *in vitro* tissue culture model.

3.2 MATERIALS AND METHODS

Tissues

Human fetal ovaries and testes were obtained following medical termination of pregnancy. Women gave consent according to national guidelines (Polkinghorne, 1989) and the study was approved by the Lothian Paediatrics / Reproductive Medicine Research Ethics Sub-Committee. Gestational age was determined by ultrasound examination prior to termination and confirmed by subsequent direct measurement of foot length (Section 2.1).

Isolation of RNA and synthesis of cDNA

Total RNA was extracted from fetal ovary and (14 - 19 weeks) and cDNA synthesis performed (Section 2.2.1)

Amplification of specific cDNAs by PCR

PCR was performed using 1µl cDNA samples, 5µM forward and reverse oligonucleotide primers and 2 x thermostart PCR mastermix in a total volume of 25µl (Section 2.2.2). Specific primers were used, and these and the resultant product sizes are given in Table 3.1 below. PCR amplification conditions for subunits and type II receptors all consisted of an initial hot start denaturation step at 95°C for 15minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds and extension at 72°C for 45 seconds. A final extension period at 72°C for 5 minutes completed the amplification. Control tubes were run in parallel for each PCR, one in which water replaced RNA and a second RT- sample in which reverse

transcriptase was omitted, to ensure there was no genomic DNA contamination. After PCR, the size of product yielded was ascertained using electrophoresis. The identity of all PCR products was confirmed by sequencing.

Lightcycler Quantitative PCR

Quantitative PCR was performed using the Lightcycler system to investigate changes in expression of the activin β A subunit over a range of gestations (Section 2.2.3). A total of 29 specimens were used, between 14 and 19 weeks gestation. Primers used, and the resultant product sizes, are given in Table 3.1.

| GENE | PRIMER SEQUENCE (5' – 3') | | PRODUCT SIZE Base pairs (bp) |
|-----------------------------|---------------------------|-------------------------|---------------------------------|
| ActRIIA | 5' | GCAAAATGAATACGAAGTCTA | 435 |
| | 3' | GCACCCTCTAATACCTCTGGA | |
| ActRIIB | 5' | CAACTTCTGCAACGAGCGCTT | 283 |
| | 3' | GCGCCCCCGAGCCTTGATCTC | |
| Inhibin α | 5' | TGAGGGCCCTGTTCTTGGATG | 278 |
| | 3' | CTGGCGGCTGCGTGTATGCTG | |
| Inhibin / activin β A | 5' | GAACTTATGGAGCAGACCTCGG | 274 |
| | 3' | TTGCCATCACACTCCAAGCC | |
| Inhibin / activin β B | 5' | GCCAGGAGCGCGTTTCCGAAATC | 325 |
| | 3' | CCGCTCGCCCCGCTCAAACAAG | |
| GAPD | 5' | GACATCAAGAAGGTGGTGAAGC | 212 |
| | 3' | GTCCACACCCTGTTGCTGTAG | |

Table 3.1. Sequences of PCR primers for inhibin / activin subunits, type II activin receptor and constitutively expressed gene GAPD.

Immunohistochemistry

Immunohistochemistry was performed on paraffin embedded sections of fetal ovary and testis to localise expression of activin and inhibin subunits and receptors, as well as follistatin, a natural antagonist to activin. Expression of activin type II receptors ActRIIA and ActRIIB, type I receptors ALK2 and ALK4 and the inhibin receptor betaglycan was also investigated (Section 2.3.1). The conditions for individual antibodies are described below. Fetal testis was used as positive control tissue in all experiments. Negative controls omitted application of primary antibody in the case of monoclonal antibodies, and substituted primary antibody incubation with non-immune serum in the case of polyclonal antibodies.

INHIBIN α SUBUNIT

After dewaxing and rehydrating, antigen retrieval was carried out. Tissue was immersed in boiling 0.01M citrate buffer pH 6.0 for 2 minutes, then cooled by addition of cold tap water. Non-specific binding was eliminated by pre-blocking with normal rabbit serum (NRS) (diluted 1:5 in TBS containing 5% bovine serum albumin). Monoclonal primary antibody to the inhibin α subunit (173.9k), gift of NP Groome (School of Biological and Molecular Sciences, Oxford Brookes University, Oxford), was used at a range of dilutions 1:1000 to 1:100 (incubated overnight at 4°C). Secondary rabbit anti-mouse biotinylated antibody was added at 1:500.

INHIBIN / ACTIVIN β SUBUNITS

Non-specific binding was eliminated by pre-blocking with NRS (diluted 1:5 in TBS containing 5% bovine serum albumin). Sections were incubated overnight at 4°C with monoclonal primary antibodies to the inhibin / activin β A subunit (E4) used at 1:1000 or β B subunit (12/13) used at 1:100, both the gift of NP Groome. Secondary rabbit anti-mouse biotinylated IgG antibody was added at 1:500. An alternative antibody directed against the β B subunit was also used, with similar results. Non-specific binding was eliminated by pre-blocking with normal swine serum (NSS) (diluted 1:5 in TBS containing 5% bovine serum albumin). Rabbit polyclonal β B (80-112)-NH₂ PBL#197, gift of W Vale (Clayton Foundation Laboratories for Peptide Biology, The Salk Institute for Biological Studies, La Jolla, California, USA), was used at and incubated overnight at 4°C. Negative controls substituted application of primary antibody. Secondary swine anti-rabbit biotinylated antibody was added at 1:500.

FOLLISTATIN

After dewaxing and rehydrating, antigen retrieval was carried out. Tissue was placed in 0.01M citrate buffer pH 6.0 and microwaved for 20 minutes, then left to stand in buffer for a further 20 minutes. Non-specific binding was eliminated by pre-blocking with NRS (diluted 1:5 in TBS containing 5% bovine serum albumin). Monoclonal antibody directed against recombinant follistatin FS288, which recognises follistatin even when bound to activin, was used at a range of dilutions 1:200 to 1:25 (incubated overnight at 4°C), gift of NP Groome (School of Biological and Molecular Sciences, Oxford Brookes

University, Oxford). Secondary rabbit anti-mouse biotinylated antibody was added at 1:500.

ACTIVIN RECEPTORS

Non-specific binding was eliminated by pre-blocking with normal goat serum (NGS) (diluted 1:5 in TBS containing 5% bovine serum albumin). Rabbit antibodies to ActRIIA and ActRIIB were kindly donated by Dr C-H Heldin (Ludwig Institute for Cancer Research, Uppsala, Sweden), and used at a concentration of 1:600 and 1:400 respectively. Rabbit antibodies to ALK2 and ALK4 were also kindly donated by Dr C-H Heldin and both used at concentration 1:150. All were incubated overnight at 4°C, and 1:500 secondary goat anti-rabbit biotinylated antibody added thereafter.

BETAGLYCAN

Non-specific binding was eliminated by pre-blocking with NRS (diluted 1:5 in TBS containing 5% bovine serum albumin). Goat antibody to betaglycan (Santa Cruz Biotechnology, CA, USA) was used at a dilution of 1:25 (incubated overnight at 4°C). Secondary rabbit anti-goat biotinylated antibody was added at 1:500.

BrdU

After dewaxing and rehydrating, antigen retrieval was carried out. Tissue was immersed in boiling 0.01M citrate buffer pH 6.0 for 2 minutes, then cooled by addition of cold tap water. Non-specific binding was eliminated by pre-blocking with NRS (diluted 1:5 in TBS containing 5% bovine serum albumin). Mouse monoclonal primary antibody

(Roche diagnostics, Lewes) was used at a dilution of 1:30, and incubated at 4°C overnight. Secondary rabbit anti-mouse biotinylated antibody was added at 1:500.

Nuclear diameter measurement

The size distribution of germ cells expressing activin β A subunit was determined in specimens of 18 – 19 weeks gestation using the Provis image analysis system (Section 2.3.2).

Primary cell culture

Ovaries from 7 fetuses, ranging from 14 to 17 weeks gestation, were dissected using a sterile technique. They were initially bisected transversely and one half further dissected to yield tissue fragments approximately 1mm^3 . The intact half ovary was immediately fixed for histological analysis. Tissue fragments were cultured for 17 hours in control media or control media supplemented with 100ng/ml recombinant human activin A (Section 2.4). BrdU was used as a marker of cell proliferation. In each experiment BrdU was present in the media of one control and one treatment group for the duration of the experiment. BrdU was added to the other control and treatment group for the final 4 hours of tissue culture only. At the end of culture, tissue was fixed, transferred to 70% ethanol and embedded in paraffin wax for histological analysis.

Tissue was sectioned at $5\mu\text{m}$ thickness and processed for immunohistochemistry to detect BrdU staining as described (Section 2.3.1). On completion of the

immunohistochemistry protocol, sections were counterstained with haematoxylin, dehydrated and mounted.

Analysis of cultured tissue

Sections of uncultured control and cultured tissue were analysed to quantify the effects of culture and activin A on the number and proliferation of germ cells (Section 2.5).

3.3 RESULTS

Expression of mRNAs for activin/inhibin subunits and receptors

PCR detected mRNA for all activin/inhibin subunits and type II receptors (Figure 3.1).

Amplicons of expected sizes corresponding to the inhibin α (278 bp), inhibin / activin β A (274 bp) and β B (325 bp) subunits and the activin receptors ActRIIA (435 bp) and ActRIIB (283 bp) were detected in ovaries studied across 14 to 19 weeks gestation.

Product identity was confirmed by direct sequencing.

Quantitative PCR

Changes in the expression of the activin β A subunit mRNA between 14 and 19 weeks gestation were investigated by quantitative PCR. There was an approximately 2-fold increase in the relative expression of the β A subunit mRNA expression between 14 and 19 weeks gestation (ANOVA, $p < 0.001$). The increase in expression was not continuous across the gestational range examined, but mostly occurred between 17 and 19 weeks (Figure 3.2).

Figure 3.1 Expression of mRNA for inhibin / activin subunits and type II activin receptors in the human fetal ovary. PCR analysis of samples extracted from human fetal ovaries at 14, 16 and 19 weeks gestation. Expression of mRNA for inhibin / activin subunits and receptors ActRIIA and ActRIIB as indicated. Lanes marked 14-, 16- and 19- contained samples in which reverse transcriptase was omitted. The size of each amplicon is indicated on the right.

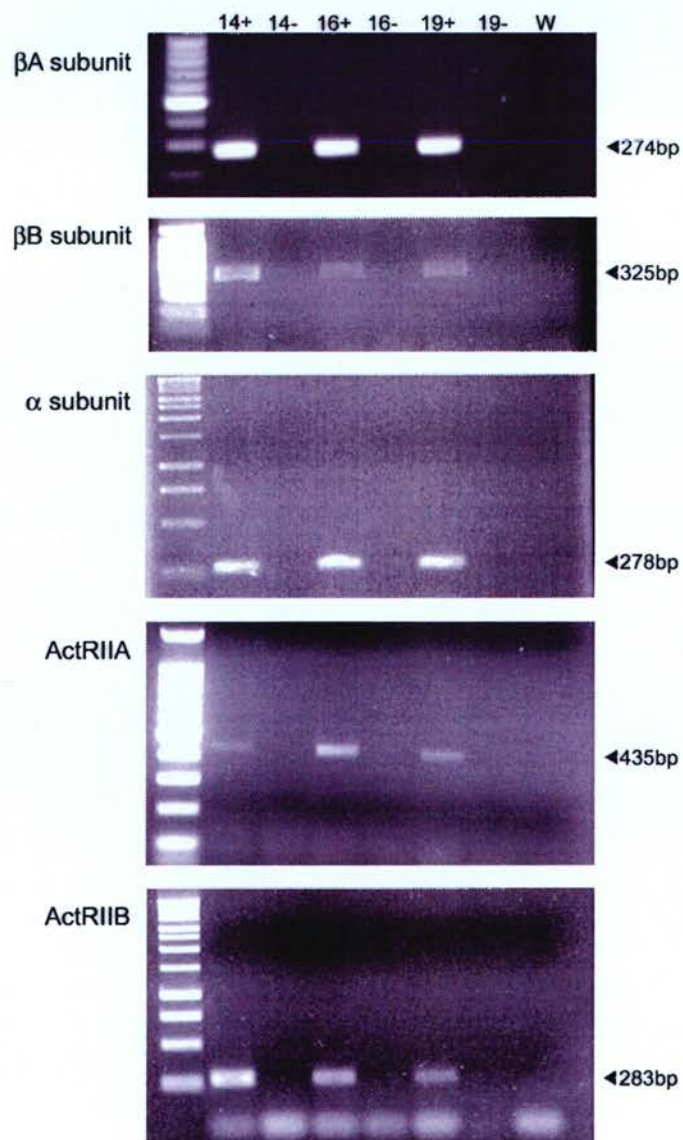
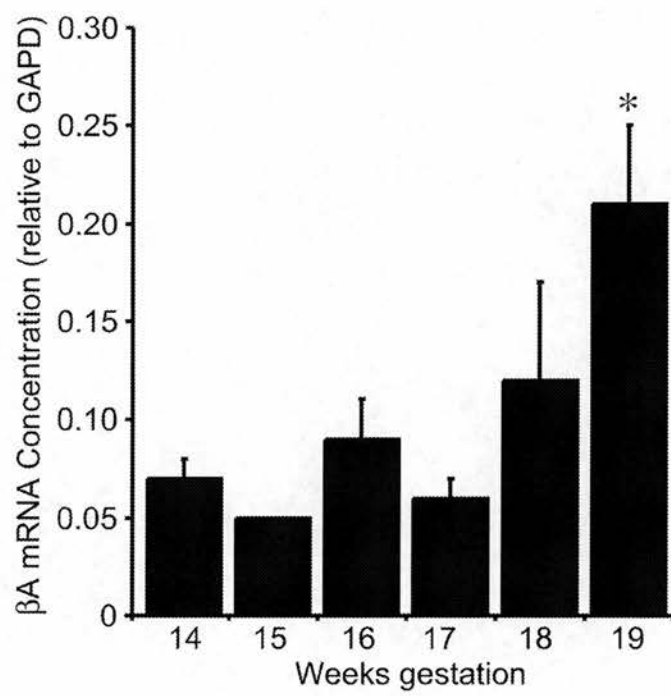


Figure 3.2 Quantitative PCR analysis of β A expression in the fetal ovary. Expression of β A subunit mRNA was quantified in human fetal ovary specimens over the gestational range 14 to 19 wk. Data calculated as β A mRNA expression relative to GAPD for ovaries of 14, 15, 16, 17, 18 and 19 wk gestation (n= 6, 5, 5, 5, 3 and 5 respectively, mean \pm SEM). *, p <0.001 by ANOVA.



Immunohistochemistry

Inhibin / Activin Subunits

Expression of the βA inhibin / activin subunit protein was detected in the fetal ovary at all gestations investigated. It was specifically localised to the germ cell cytoplasm at all gestations studied, but a marked change in the pattern of immunostaining was seen across the gestational range (Figure 3.3 D-F). At 14 weeks gestation a gradient of distribution was observed. Oogonial immunostaining was detected throughout the ovary, but was most marked at the periphery particularly in germ cells in the outermost few layers of the gonad. At 17-19 weeks gestation a different distribution of immunostaining had emerged with a discrete sub-population of germ cells showing intense staining. These germ cells tended to be larger oocytes, and were distributed in small clusters of less than ten largely located in the medullary region of the ovary. However, oocytes within newly formed primordial follicles showed only weak or no immunostaining for activin βA .

Differential expression of βA subunit by larger germ cells was confirmed by direct measurement of nuclear diameter. A total of 527 germ cells were measured, of which 79 (15%) were intensely immunopositive for the βA subunit. These βA -expressing germ cells were significantly larger than those only weakly immunopositive or immunonegative ($11.3\mu\text{m} \pm 0.2$ vs $9.8\mu\text{m} \pm 0.1$, $p=0.0001$) although the very largest germ cells with nuclear diameter $>16\mu\text{m}$, i.e. those within primordial follicles, were immunonegative (Figure 3.4).

Expression of β B inhibin / activin subunit protein was also detected throughout the ovary across the range of gestations examined. Immunostaining was widespread, with both germ cells and stromal cells expressing the β B subunit (Figure 3.3 G-I). However at the periphery of the ovary some germ and somatic cells did not express the β B subunit. There was no difference in distribution of immunostaining of either stromal or germ cells between 14 and 18 weeks gestation.

Immunostaining for the inhibin α subunit was not detected in any cell type in the human fetal ovary across a range of gestations and despite using a wide range of antibody titres. Fetal testes was used as a positive control and showed immunostaining in both Leydig cells in the interstitium and Sertoli cells in the seminiferous tubules as previously described (Figure 3.3 A-C) (Anderson *et al.*, 2002a).

Figure 3.3 Immunohistochemical localization of inhibin / activin subunits in human fetal ovary.

A, inhibin α subunit, 14 week ovary. **B**, inhibin α subunit, 19 week ovary. **C**, inhibin α subunit positive control, 19 week testis. **D**, β A subunit, 14 week ovary. **E**, β A subunit, 17 week ovary (inset shows negative control). **F**, β A subunit, 17 week ovary at higher magnification. **G**, β B subunit, 14 week ovary. **H**, β B subunit, 18 week ovary (inset shows negative control). **I**, β B subunit, 18 week ovary at higher magnification. Positive staining in all panels is brown and sections are counterstained with haematoxylin.

o Oogonium; **s** Stroma; **t** Tubule; **i** Interstitium.

Scale bars in **A**, **B**, **C**, **D**, **E**, **G** and **H**, 50 μ m; **F** and **I**, 20 μ m

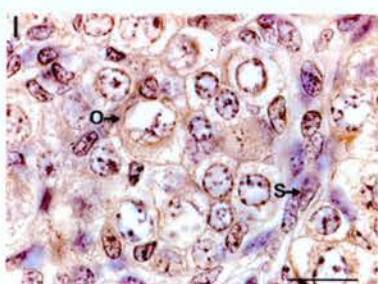
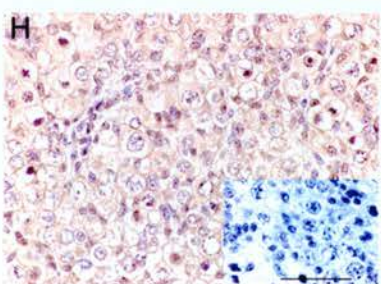
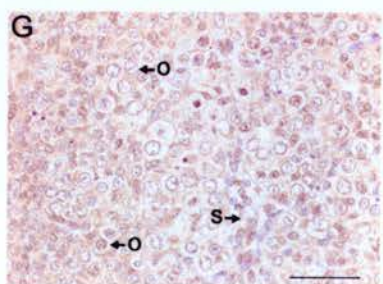
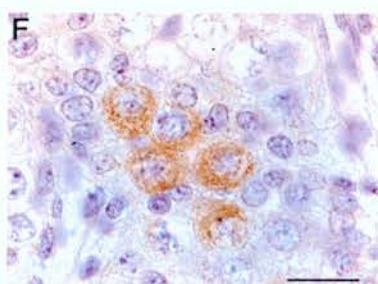
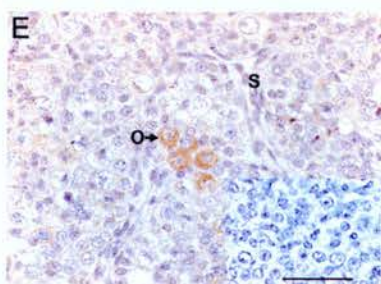
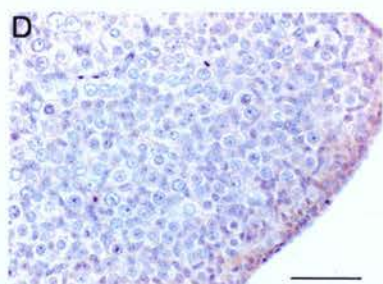
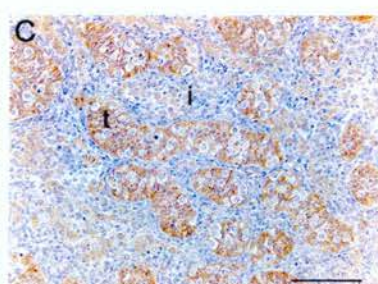
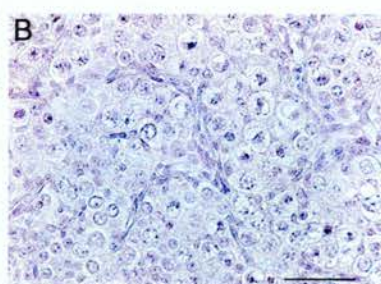
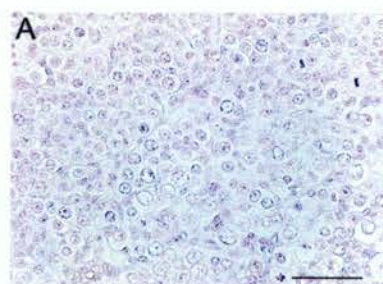
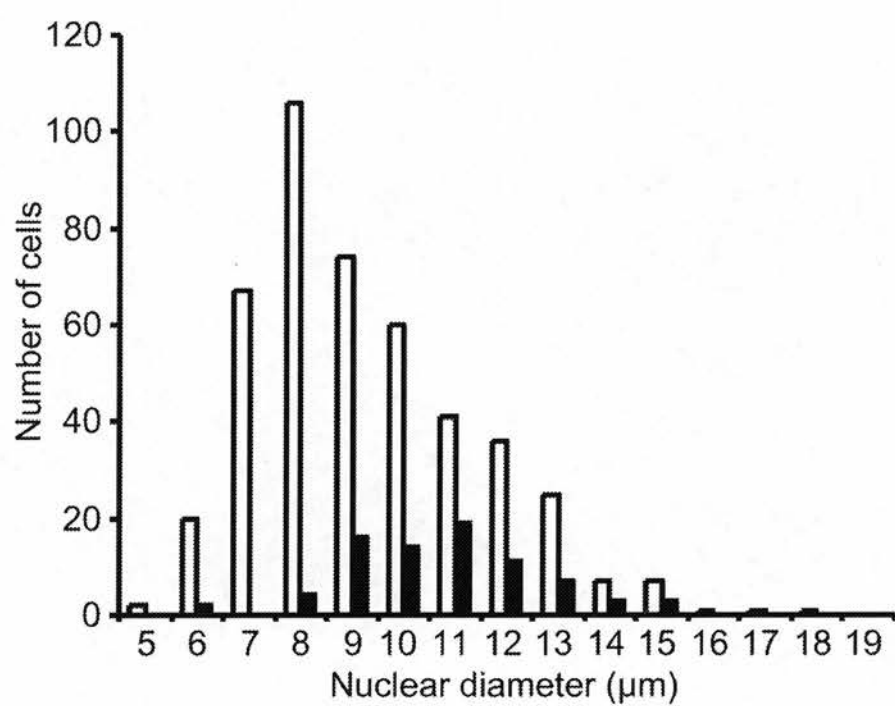


Figure 3.4 Size distribution of β A immunopositive germ cells in the human fetal ovary. Histogram shows the frequency of distribution of nuclear diameter of cells strongly immunopositive for β A subunit (filled columns) and those either weakly immunopositive or immunonegative for β A subunit (open columns) in human fetal ovary at 18 – 19 weeks gestation, n = 79 and n = 448 respectively.



Activin and inhibin receptors

Expression of the type II receptor ActRIIA was detected at all gestations from 14 to 21 weeks and was localised specifically to germ cell cytoplasm (Figure 3.5 C-D). There was no immunostaining in stromal cells or the pre-granulosa cells of primordial follicles, nor cells at the ovarian surface. There was no significant difference in distribution across the gestational range studied.

ActRIIB immunoexpression was distributed widely within the fetal ovary at all gestations from 14 weeks to 21 weeks (Figure 3.5 E-F). Immunostaining was detected in both stromal and germ cells but not in the cells at the ovarian surface. Intense immunostaining was noted in larger germ cells at later gestations and expression persisted in cytoplasm of oocytes organising into primordial follicles.

Expression of ALK4 protein, the preferential activin type I receptor, was detected throughout the ovary predominantly in stromal and pre-granulosa cells but also with weak expression in germ cells (Figure 3.5 A). At 14 weeks gestation, ALK4 immunostaining clearly demarcated a meshwork of ovarian stromal cells with weaker immunostaining of the oogonia intermingled with more strongly immunopositive pre-granulosa cells. At later gestations (18 weeks) the ovarian stroma was less strongly immunopositive, and the predominant site of ALK4 expression was the pre-granulosa cells surrounding individual and grouped oogonia, although oogonia remained weakly immunopositive.

In contrast to ALK4, expression of ALK2 protein was exclusively localised to the stromal cells at 14 to 18 weeks gestation, with no immunostaining identified in germ cells at any gestation (Figure 3.5 B). Intense immunostaining was particularly notable in many pre-granulosa cells between and outlining germ cells. This pattern of stromal cells immunostaining appeared to increase with increasing gestation and the formation of primordial follicles.

No expression of β -glycan protein was detected in any mid-trimester gestation ovary studied. Intense immunostaining was detected in the peritubular cells of fetal testes used as a positive control (Figure 3.5 G-H).

In all cases negative controls showed no immunostaining. Fetal testis sections were used as a positive control, and showed distinct immunostaining as previously described (Anderson *et al.*, 2002a).

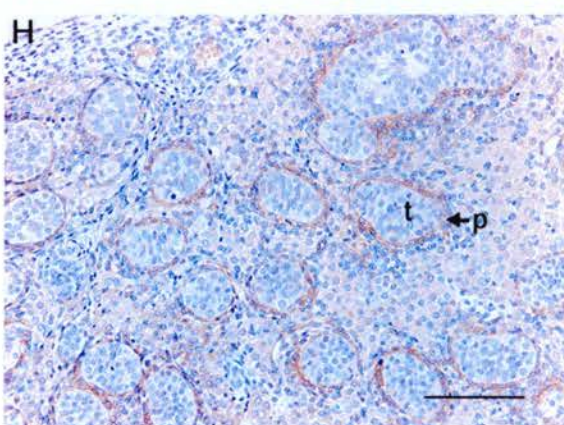
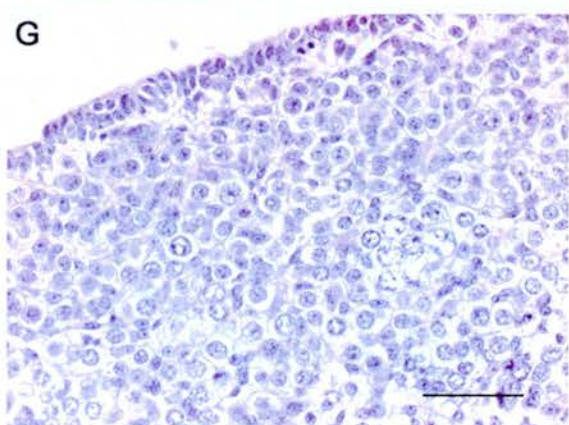
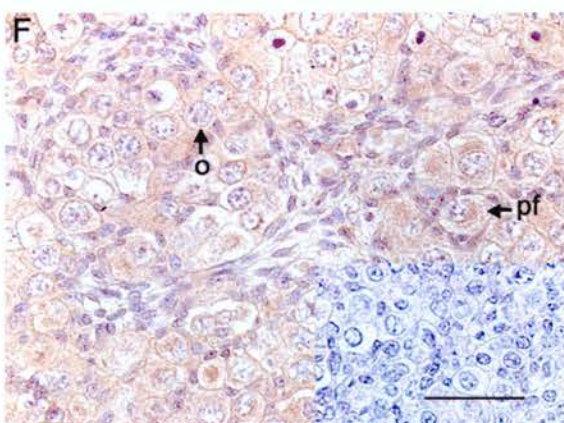
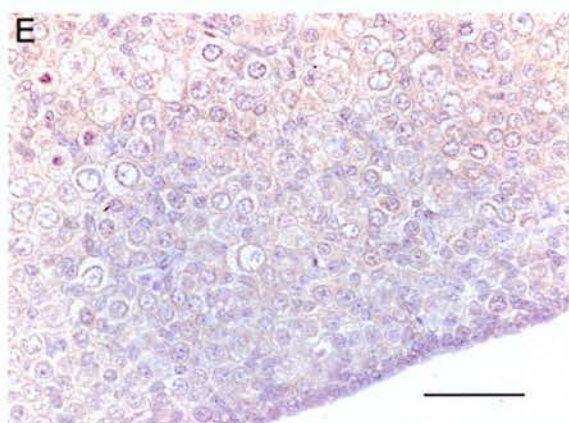
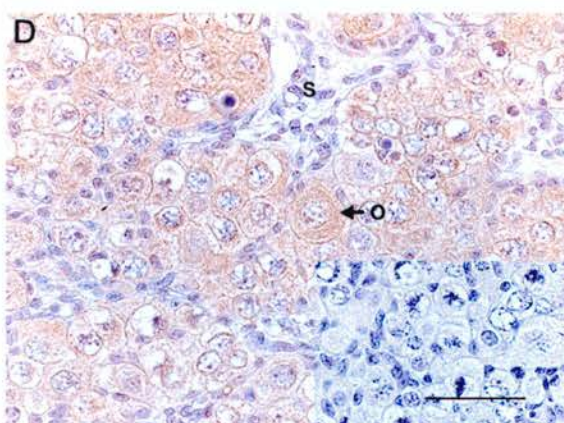
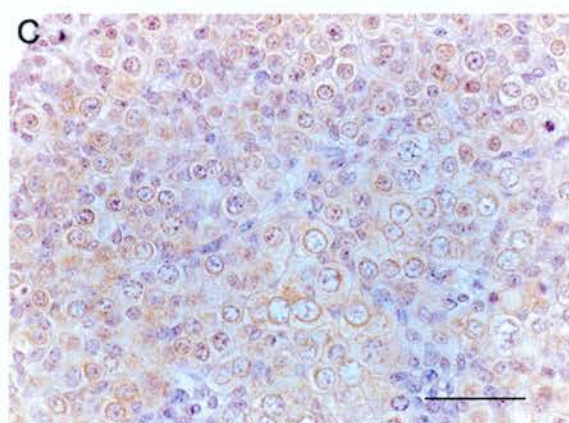
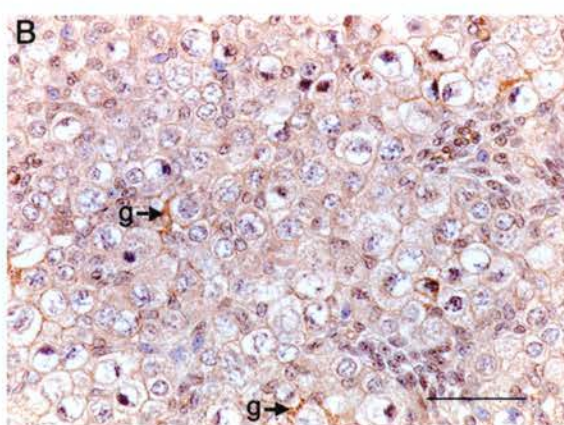
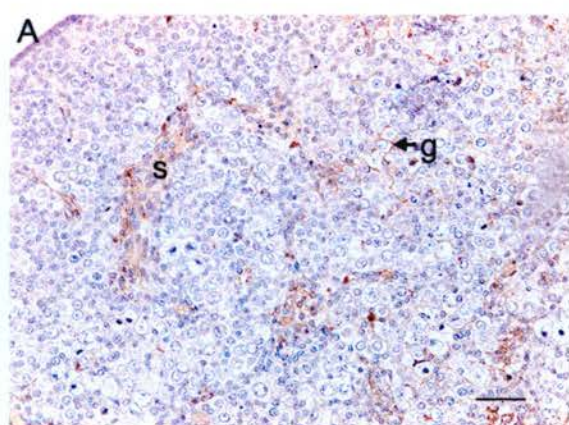
Figure 3.5 Immunohistochemical localization of inhibin / activin receptors in human fetal ovary.

A, ALK2, 14 week ovary. **B**, ALK4, 18 week ovary. **C**, ActRIIA, 14 week ovary. **D**, ActRIIA, 21 week ovary (inset shows negative control). **E**, ActRIIB, 14 week ovary. **F**, ActRIIB, 21 week ovary (inset shows negative control). **G**, betaglycan, 14 week ovary. **H**, betaglycan positive control, 15 week testes.

Positive staining in all panels is brown and sections are counterstained with haematoxylin.

s Stroma; **g** Pre-granulosa cell; **o** Oogonium; **pf** Primordial Follicle; **t** Tubule; **p** Peritubular cells.

Scale bars: 50µm in all panels.



Tissue Culture

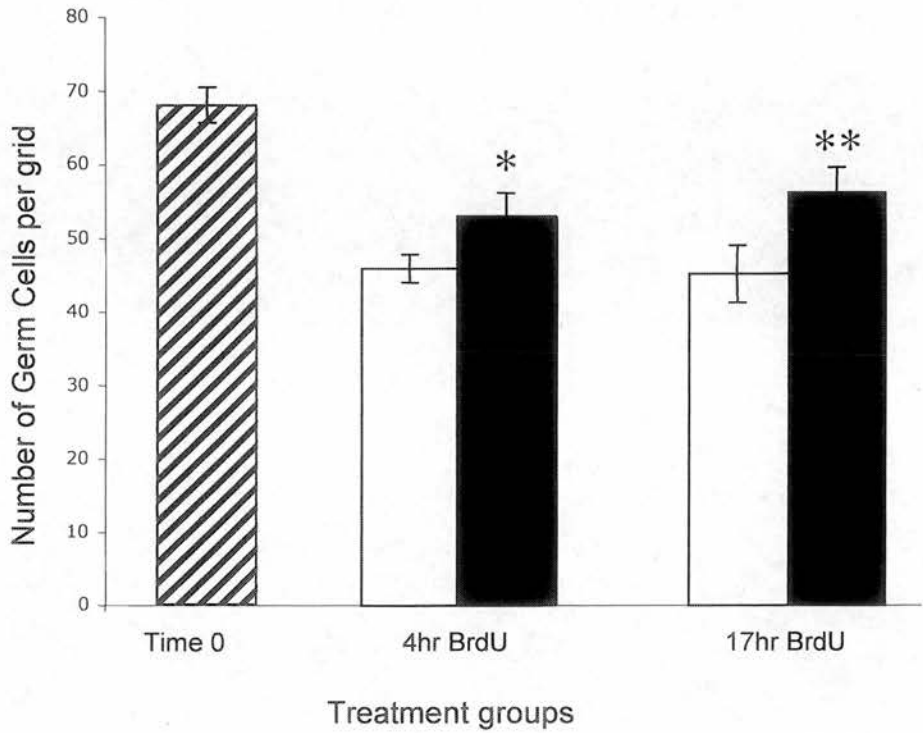
A total of 7 separate experiments were carried out to investigate the effect of activin on germ cell survival and proliferation *in vitro*, using ovaries between 14 and 17 weeks gestation. Comparison of germ cell number in control cultures showed good oögonial survival (46 ± 2 oocytes per grid after 17 hr culture vs 68 ± 2 in uncultured and undissected tissue) and there was no effect of changing the medium 4 hours before the end of the culture period in those experiments where BrdU was added at that time point (45 ± 4 germ cells per grid in controls vs 46 ± 3 in those in which the medium was changed). Treatment with 100ng/ml activin A resulted in a significant increase in total number of germ cells. Activin A treated groups showed a mean increase of 20% in the total number of germ cells from 45 ± 4 to 56 ± 4 per grid, $p=0.001$. Very similar results were obtained in the cultures in which BrdU was present for 4 hours (Figure 3.6 A). When BrdU was present in the culture medium for the duration of culture (17 hours), more germ cells were immunopositive than when BrdU was present for only the final 4 hours of culture, as expected (6.4 ± 1 vs 2.8 ± 0.4 stained germ cells per grid when exposed to BrdU for only 4 hours). Activin A treatment also resulted in a significant increase in number of BrdU immunopositive germ cells. There was a 44% increase in number of immunostained germ cells (9.2 ± 1.3 vs 6.4 ± 0.8 per grid, $p=0.01$) when cultured with activin A and BrdU for 17 hours. Immunostained germ cells were increased by 89% (5.3 ± 0.9 vs 2.8 ± 0.4 per grid, $p<0.05$) when cultured with activin A for 17 hours and exposed to BrdU for only the final 4 hours of culture (Figure 3.6 B, Figure 3.7).

Figure 3.6 Analysis of the effect of activin A on the number of germ cells in human fetal ovary.

A, Number of germ cells per grid for time 0 tissue (shaded column), and groups cultured for 17 hours without (control groups, open columns) or with 100ng/ml recombinant activin A (filled columns) (mean \pm SEM). Student's t-test * $p < 0.05$; ** $p = 0.001$ vs control.

B, Number of BrdU immunopositive germ cells per grid: control groups (open columns), with 100ng/ml recombinant activin A (filled columns) (mean \pm SEM). In both A and B treatment and control groups were exposed to BrdU for either 4 hours or for the duration of culture (17 hours). Student's t-test *, $p < 0.05$; **, $p = 0.01$ vs control.

A



B

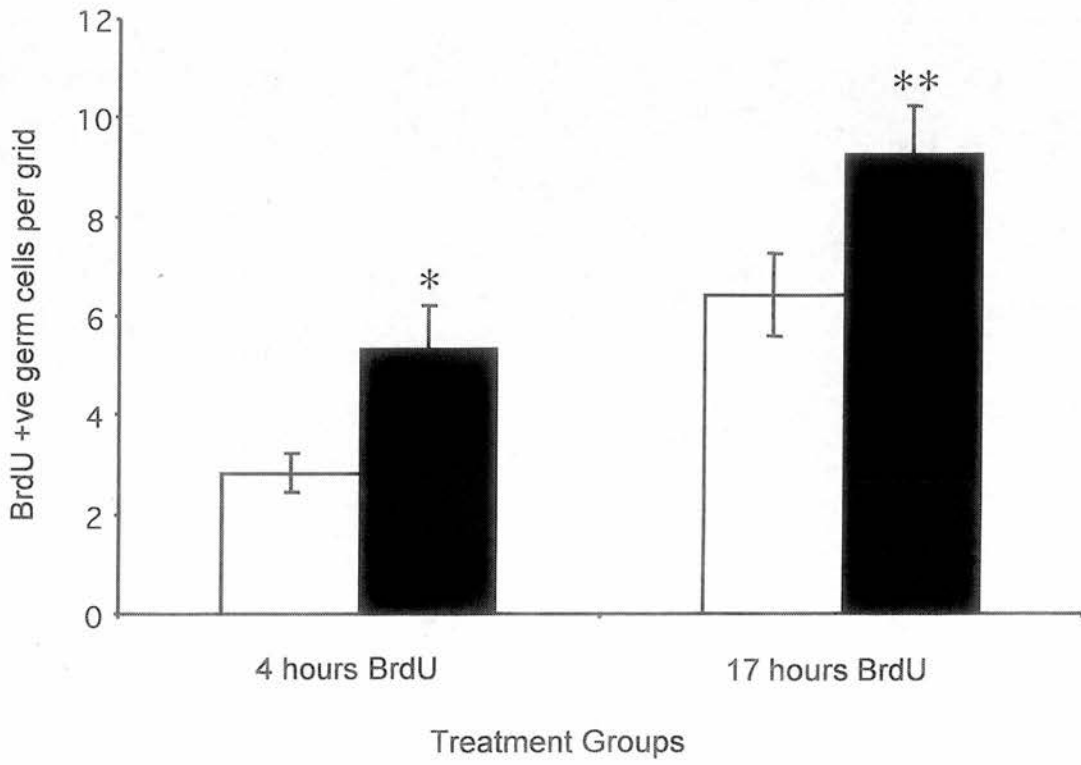
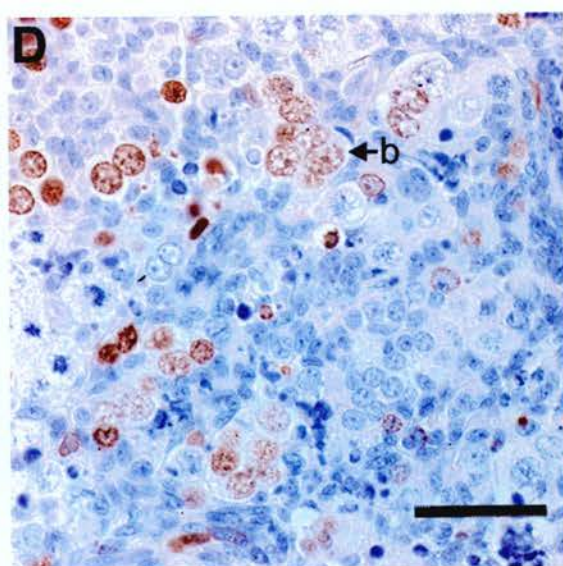
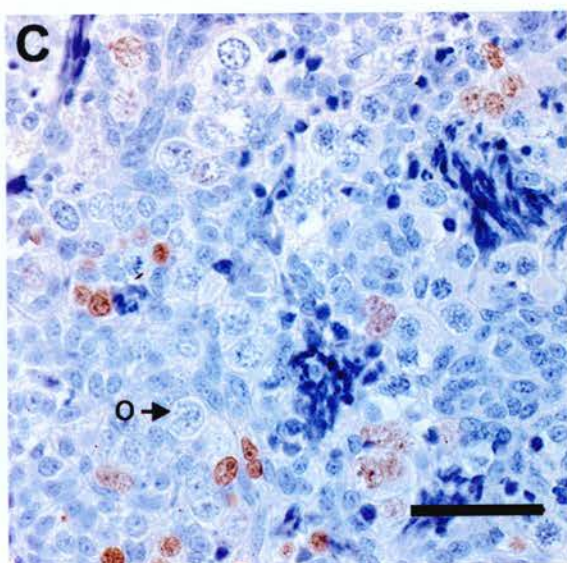


Figure 3.7 Representative photomicrographs of control (C) and activin-treated (D) 17 week human fetal ovary cultured with BrdU for 17 hr, and immunostained for BrdU. Positive staining in all panels is brown and sections are counterstained with haematoxylin.

o Oogonium; **b** BrdU-immunopositive oogonium.

Scale bar: 50µm.



3.4 DISCUSSION

High interspecies conservation of activins, inhibins and activin receptors and the universal presence of activins in mammals, birds, amphibians and fish suggest an evolutionarily conserved role of these proteins in animal development. Activins have potent effects on early amphibian embryonic events during mesoderm-forming (Nieuwkoop) induction both *in vitro* and *in vivo* (Dyson and Gurdon, 1997). In mammals, activins have also been demonstrated to have morphogenetic effects, notably in renal tubule development (Ritvos *et al.*, 1995). However, there are few published data available concerning the presence, expression and potential roles of activins, inhibins and their receptors during mammalian gonadal development. Whilst activin is thought to act primarily in an autocrine and/or paracrine manner, inhibin acts primarily as an endocrine feedback regulator of pituitary FSH release (Welt *et al.*, 2002). Our data demonstrate the presence and localisation of activin receptors and subunits within the developing human ovary, and suggest a role for activin A in the regulation of germ cell proliferation during the developmental period leading up to primordial follicle formation.

Transgenic mice have been generated in order to study physiological models deficient in inhibins, activins and their receptors (Burns and Matzuk, 2002). Gene knockout experiments have been inconclusive in determining activin function *in vivo*, but functional analysis would suggest that activins are not essential for mammalian mesoderm formation. A number of reasons are cited to explain the lack of definitive conclusions from this body of work; some knockout mutations are lethal, deficient

activin ligand or receptor signalling results in a phenotype with suppressed FSH and consequential reproductive and developmental defects, and there may be some functional overlap between TGF β superfamily ligand and receptor signalling. Mutations in the gene encoding the β A subunit are lethal. Mutant mice develop to term but have secondary palate defects and lack whiskers and lower incisors (Matzuk *et al.*, 1995b). The mutant mice die within 24 hours of birth due to feeding difficulties and there are thus no conclusions regarding ovarian follicular development. Mice lacking the β B subunit suffer eye lesions as a result of failed eyelid fusion. Mutant females do not show a disruption in follicular development but manifest impaired reproduction characterised by perinatal lethality of their offspring (Vassalli *et al.*, 1994). Highly increased β A expression was noted in the ovaries of these β B deficient female mice, suggesting the possibility of functional compensation within the TGF β superfamily. Using a gene knockin approach, it has been found that activin β B can rescue the craniofacial defects and neonatal lethal phenotype of β A deficient mice. However activin β B is unable to substitute for all the functions of β A as other phenotypic abnormalities in the gonads and external genitalia, as well as delayed lethality, remain in these mice (Brown *et al.*, 2000). There is also some evidence that it is the second messenger (Smad) signalling that is more crucial to specific embryonic and extra-embryonic development pathways than particular ligand and receptor interactions (Chang *et al.*, 2001). Lastly, it is interesting to note that mice carrying a null mutation in the ActRII gene do not phenocopy activin-deficient mice, although they suffer defective reproductive performance as a result of diminished FSH levels (Matzuk *et al.*, 1995a).

Activin subunits and receptors showed a differential distribution within the developing ovary. Activins bind to a type II receptor (ActRIIA or ActRIIB), which recruits and phosphorylates a type I receptor with subsequent modulation of gene expression via Smad protein activation (Massagué, 1998). Several type I receptors, termed ALKs have been identified. ALK4 (ActRIB) is believed to be the preferential activin receptor. The activin β A subunit was specifically expressed by germ cells, whereas both somatic and germ cells expressed the β B subunit. ActRIIA was specifically localised to germ cells, whereas ActRIIB and ALK4 were both expressed by stromal and germ cells. The distinct distribution of both activin subunits and receptor subtypes between germ cells and somatic cells suggest that activin may have several distinct roles in ovarian development and organisation, and more specifically in germ cell regulation. This is supported by observations of an increase in β A mRNA expression as well as changes in the pattern of β A protein expression across the mid-trimester. With increasing gestation, intense β A expression was found in small clusters of larger, more mature germ cells. However the largest oocytes, within primordial follicles, show little or no β A expression. This pattern suggests that the increase in β A subunit expression may be transient as the germ cells mature, with reduction of gene expression following primordial follicle formation. Increased expression may, however, be associated with differences in subsequent survival, consistent with the effects of activin on oogonal survival as demonstrated in the tissue culture experiments here described.

Despite detecting inhibin α subunit mRNA by PCR, we were unable to demonstrate the protein by immunohistochemistry. Previous studies suggest that activin but not inhibin is present in the human fetal ovary at mid-gestation (16 – 23 weeks) (Rabinovici *et al.*, 1991). Rabinovici *et al* localised the β A subunit to primordial follicles by immunohistochemistry, but did not observe immunostaining for β B or α subunits. In contrast, immunostaining for all three subunits was detected in late gestation fetal rhesus monkey ovary, which contained primordial and early growing follicles. Furthermore, culture medium from mid-gestation human fetal ovaries was found to contain undetectable levels of inhibin- α by radioimmunoassay. Recent immunolocalisation studies performed in baboons revealed minimal to non-detectable levels of α subunit in mid and late gestation ovaries (Billiar *et al.*, 2003) and there is also indirect evidence from *in situ* hybridisation studies on primate ovaries that follicles preferentially synthesise activin rather than inhibin in the early stages of development (Schwall *et al.*, 1990; Yamoto *et al.*, 1993). The putative inhibin receptor betaglycan (Lewis *et al.*, 2000) was also not detected in the midtrimester human fetal ovary. The fact that neither the α subunit nor betaglycan receptor were identified by immunohistochemistry in mid-trimester human fetal ovaries indicates that inhibin is not a regulatory factor at this stage of development, but becomes of importance later during follicular growth (Welt *et al.*, 2002).

An *in vitro* tissue culture model was used to investigate the effect of activin on oogonal survival and proliferation. Activin A treatment resulted in an increase in germ cell

proliferation and survival in fetal ovary tissue fragments. This concurs with the earlier demonstration that activin A stimulated [^3H] thymidine incorporation *in vitro* in the differentiating rat ovary (Kaipia *et al.*, 1994). BrdU was used as a marker for cell proliferation. It is interesting to note that immunostaining was observed predominantly towards the edge of cultured ovarian fragments. This may represent a diffusion effect, but may also partly reflect the organisation of the tissue. Mitotically dividing germ cells tend to be placed peripherally in the mid-trimester fetal ovary with a gradient of maturity across the developing ovary such that relatively more mature cells are found in the medulla compared to the cortex (Byskov, 1986). A recent study of follicle formation during fetal development in sheep also noted a cortical pattern of distribution of BrdU staining following *in vivo* administration (Sawyer *et al.*, 2002), which became more pronounced up to primordial follicle formation.

In the adult ovary, activin A induces expression of genes signalling cellular differentiation and maturation. In the developing fetal ovary key events involve germ cell proliferation, organisation and survival by somatic cell interaction and primordial follicle formation (Hirshfield, 1991; McLaren, 1991). The present data support a role of activin A in germ cell survival and proliferation. The Bcl-2 family of proteins are key regulators of apoptosis in many cell types. This family comprises pro-apoptotic (Bax, Bad and Bak) and anti-apoptotic (Bcl-2, Bcl-X_L and Mcl-1) subfamilies. Mcl-1 is a rapidly inducible short-term effector of cell viability and is expressed in a subset of oocytes at the time of primordial follicle formation (Hartley *et al.*, 2002). The

distribution of Mcl-1 in the fetal ovary is similar to that of activin β A subunit and it is possible that activin A promotes cell survival by signalling via Mcl-1, as has been demonstrated in a leukaemia cell line (Fukuchi *et al.*, 2001). While both activin A and Mcl-1 expression are most marked in larger oogonia preceding primordial follicle expression, oocytes within primordial follicles continue to express Mcl-1 whereas activin β A expression is low once that developmental stage is reached. It is possible that activin and Mcl-1 constitute part of the signalling pathway that determines survival for a small proportion of the oogonia in the mid trimester ovary.

In conclusion, the human fetal ovary is a site of expression of activin subunits and receptors prior to the formation of primordial follicles. Protein expression of the inhibin α subunit and the betaglycan receptor was not detected. The distribution of activin subunits and receptors suggests that whilst somatic cells may produce any of the activin forms, oogonia only produce activin A, and is consistent with an autocrine and/or paracrine mode of action for activins within the developing ovary. Increased expression of β A by larger/more mature oogonia may reflect selection for follicle formation and data from tissue culture experiments supports activin A as a regulatory factor in fetal germ cell proliferation and survival. These data suggest that activin may be of particular importance during the period of development immediately preceding primordial follicle formation.

CHAPTER 4

THE EFFECT OF NEUROTROPHINS ON GERM CELL SURVIVAL AND PROLIFERATION

4.1 INTRODUCTION

Mammalian ovarian development is characterised by a sequential pattern of mitotic proliferation of oogonia, initiation then arrest of meiosis, and primordial follicle formation. Waves of atresia of oogonia have been identified at particular stages of development (Baker, 1963; Speed, 1988; Pepling and Spradling, 2001) but the factors and pathways determining whether oocytes survive or die are largely unknown. Adult reproductive potential is determined by presence of primordial follicles, which offer a source of oocytes to mature, ovulate and fertilise. Thus, early regulation of germ cell proliferation and survival in the form of primordial follicles is crucial to human reproductive potential.

Neurotrophins are a small family of closely related peptide factors. NGF was the first to be discovered; BDNF, NT3, and NT4 have since been identified in mammals (Snider, 1994). The neurotrophins bind selectively to glycoprotein tyrosine receptor kinases, Trk receptors, which consist of an extracellular domain containing the neurotrophin binding site, a short transmembrane sequence and an intracellular domain that encodes a tyrosine kinase. There are three members of the Trk receptor family: Trk A, the receptor for NGF, TrkB, the receptor for BDNF and NT4 and TrkC, the receptor for NT3. Truncated isoforms of TrkB and TrkC, which lack the intracellular tyrosine kinase domains, have also been identified (Klein *et al.*, 1990; Dechant, 2001). In addition to the Trk receptors, all neurotrophins bind to a membrane receptor known as p75, a member of the tumour necrosis receptor superfamily. Effects of neurotrophins on cell survival and growth are thought to be mediated by the Trk receptors, which form homodimers on ligand binding

and autophosphorylate to trigger intracellular signalling cascades (Segal and Greenberg, 1996). Ligand binding activates Trk receptors and initiates a signalling cascade resulting in either neuronal differentiation or mitogenic stimulation (Kaplan and Stephens, 1994).

The neurotrophin family are known to have a critical role in cell proliferation, migration, differentiation and survival in the nervous system (Henderson *et al.*, 1993; Davies, 1994; Snider, 1994). BDNF promotes maturation of sensory neurons before they become dependent on neurotrophins for survival (Wright *et al.*, 1992), and can stimulate differentiation of embryonic neural crest cells (Kalcheim and Gendreau, 1988; Sieber-Blum, 1991). Neurotrophins also have important functions in non-neuronal tissue, including the mammalian ovary where they appear to be involved in folliculogenesis (Dissen *et al.*, 1995; Dissen *et al.*, 2001; Romero *et al.*, 2002) and ovulation (Dissen *et al.*, 1996; Mayerhofer *et al.*, 1996), as well as oocyte maturation (Seifer *et al.*, 2002a; De Sousa *et al.*, 2004; Kawamura *et al.*, 2005; Martins da Silva *et al.*, 2005). BDNF is secreted by cumulus granulosa of the human ovarian follicle (Seifer *et al.*, 2002a; Seifer *et al.*, 2006) and is a physiological growth factor in follicular fluid (Seifer *et al.*, 2003).

NT4 and BDNF are also expressed in the developing mammalian ovary (Spears *et al.*, 2003). Initially NT4 is present in oocytes, but this site of expression predominantly switches to granulosa cells at, or around the time of, primordial follicle assembly (Anderson *et al.*, 2002a). In rats, expression of TrkB (the preferential receptor for NT4 and BDNF) mRNA increases sharply during the period of follicle formation (Dissen *et*

al., 1995). Thereafter, TrkB receptors are expressed at low, and seemingly unchanging levels in oocytes and granulosa cells of both primordial and growing follicles. Truncated TrkB appears to be selectively expressed in oocytes, and targeted to the cell membrane as primary follicles initiate growth (Paredes *et al.*, 2004). Expression and distribution of NT4 and BDNF in the human fetal ovary during the period of oogonial proliferation leading up to primordial follicle formation was investigated. Localisation of TrkB, truncated TrkB and p75 receptors during this period of ovarian development was also explored, in order to identify potential sites of action of these regulatory factors. It is hypothesised that TrkB signalling is important in primordial follicle assembly and / or oocyte survival, and as oogonia in the mid-trimester human fetal ovary express the TrkB receptor, the effect of NT4 and BDNF on germ cell survival and proliferation was investigated using an *in vitro* tissue culture model. In addition to treatment with the two ligands, the effect of removal of the influence of endogenous NT4 was investigated using an antibody against NT4. Furthermore, co-incubation of the antibody treatment with either NT4 or BDNF supplements investigated the effects of ligand replacement.

4.2 MATERIALS AND METHODS

Tissues

Human fetal ovaries were obtained following medical termination of pregnancy.

Women gave consent according to national guidelines (Polkinghorne, 1989) and the study was approved by the Lothian Paediatrics / Reproductive Medicine Research Ethics Sub-Committee. Gestational age was determined by ultrasound examination prior to termination and confirmed by subsequent direct measurement of foot length (Section 2.3).

Immunohistochemistry

Immunohistochemistry was performed on paraffin embedded sections of fetal ovary to localise expression of NT4 and BDNF. Expression of TrkB, truncated TrkB and p75 receptors was also investigated (Section 2.3.1). The conditions for individual antibodies are described below. Rat brain (donated from Dr P Saunders, MRC Human Reproductive Sciences Unit, Centre for Reproductive Health, Edinburgh) was used as positive control tissue. Negative controls omitted application of primary antibody in the case of monoclonal antibodies, and substituted primary antibody incubation with non-immune serum in the case of polyclonal antibodies.

NT4

Non-specific binding was eliminated by pre-blocking with NSS (diluted 1:5 in TBS containing 5% bovine serum albumin) for 30 minutes at room temperature. Rabbit polyclonal primary antibody (Santa Cruz, CA, USA) was added at 1:100 (in diluted

swine serum) and incubated at 4°C overnight. Secondary swine anti-rabbit biotinylated IgG antibody was subsequently added at 1:500 (in dilute swine serum, 30 minutes, room temperature).

BDNF

After dewaxing and rehydrating, antigen retrieval was carried out. Tissue was immersed in boiling 0.01M citrate buffer, pH 6.0, for 2 minutes, and then cooled by addition of tap water. Non-specific binding was eliminated by pre-blocking with NSS (diluted 1:5 in TBS containing 5% bovine serum albumin) for 30 minutes at room temperature. Rabbit polyclonal antibody (Santa Cruz, CA, USA) was added at 1:50 (diluted in swine serum) and incubated overnight at 4°C. Secondary swine anti-rabbit biotinylated IgG antibody was added at 1:500 (in dilute swine serum, 30 minutes, room temperature).

TrkB

After dewaxing and rehydrating, antigen retrieval was carried out by immersing in boiling 0.01M citrate buffer, pH 6.0, for 2 minutes. Tissue was then cooled by addition of tap water. Non-specific binding was eliminated by pre-blocking with normal donkey serum (diluted 1:5 in TBS containing 5% bovine serum albumin) for 30 minutes at room temperature. Chicken polyclonal antibody (Promega, Southampton, UK) was added at 1:25 (diluted in donkey serum) and incubated at 4°C overnight. Secondary donkey anti-chicken biotinylated IgG antibody (Jackson laboratories, Bar Harbor, ME) was subsequently added at 1:500 (in dilute swine serum, 30 minutes, room temperature).

Truncated TrkB

After dewaxing and rehydrating, antigen retrieval was carried out by immersing tissue sections in boiling 0.01M citrate buffer, pH 6.0, for 2 minutes, then cooling by addition of tap water. Non-specific binding was eliminated by pre-blocking with NSS (diluted 1:5 in TBS containing 5% bovine serum albumin) for 30 minutes at room temperature. Rabbit polyclonal antibody (Santa Cruz, CA, USA) was added at 1:50 or 1:100 (diluted in swine serum) and incubated overnight at 4°C. Secondary swine anti-rabbit biotinylated IgG antibody was subsequently added at 1:500 (in dilute swine serum, 30 minutes, room temperature).

p75

After dewaxing and rehydrating, antigen retrieval was carried out. Tissue was immersed in boiling 0.01M citrate buffer, pH 6.0, for 2 minutes and then cooled by addition of cold tap water. Non-specific binding was eliminated by pre-blocking with NRS (diluted 1:5 in TBS containing 5% bovine serum albumin). Mouse monoclonal primary antibody (Strattech Scientific Ltd., Soham, Cambridgeshire, UK) was used at a dilution of 1:30, and incubated at 4°C overnight. Secondary rabbit anti-mouse biotinylated antibody was added at 1:500.

BrdU

Antigen retrieval was carried out by immersion in boiling 0.01M citrate buffer, pH 6.0, for 2 minutes, then cooling by addition of cold tap water. Non-specific binding was eliminated by pre-blocking with NRS (diluted 1:5 in TBS containing 5% bovine serum

albumin). Mouse monoclonal primary antibody (Roche Diagnostics, Lewes, UK) was used at a dilution of 1:30, and incubated at 4°C overnight. Secondary rabbit anti-mouse biotinylated antibody was added at 1:500.

Primary tissue culture

In five separate experiments, ovaries from fetuses, ranging from 15 to 17 weeks gestation, were dissected using a sterile technique to yield tissue fragments approximately 1mm³. Several pieces were randomly chosen and immediately fixed for histological analysis. Tissue fragments were cultured for 24 hours in control media or control media supplemented with 100ng/ml NT4 (Peprotech EC), 10µg/ml antiserum directed against NT4 (anti-NT4), 100ng/ml NT4 and 10µg/ml anti-NT4, 100ng/ml BDNF (Peprotech EC) or 100ng/ml BDNF and 10µg/ml anti-NT4 (Section 2.4). BrdU was used in all groups as an indicator of cell proliferation at a concentration of 10µl/ml. At the end of culture, tissue was fixed in Bouin's fluid for 1 hour, transferred to 70% ethanol followed by processing into paraffin using standard methods.

Tissue was sectioned at 5µm thickness and processed for immunohistochemistry to detect BrdU staining as described (Section 2.3.1). On completion of the immunohistochemistry protocol, sections were counterstained with haematoxylin, dehydrated and mounted.

Analysis of cultured tissue

Using stereology, sections of uncultured control and cultured tissue were analysed to quantify the effects of culture and NT4, BDNF, anti-NT4 or a combination of NT4 and anti-NT4 and BDNF and anti-NT4 on the number and proliferation of germ cells (Section 2.5). Analyses were performed blinded to the treatment group.

4.3 RESULTS

Immunohistochemistry

NT4, as well as TrkB and p75 receptor protein expression was detected by immunohistochemistry across the mid-trimester. In addition, BDNF was detected by immunohistochemistry at 14 - 19 weeks gestation.

Oogonia showed weak cytoplasmic immunostaining for NT4 at all gestations. Intense staining was detected in pregranulosa cells among and immediately surrounding the nests of germ cells, outlining the germ cells both individually and in clusters. Stromal cells also showed slight staining, although this was more marked in cells at the edge of the stroma and in close proximity to oogonial clusters. The most intense expression of NT4 protein was seen in the cytoplasm of flattened granulosa cells of primordial follicles at later gestations. This pattern of staining clearly demarcated primordial follicles predominantly located in the medullary region of the ovary from the more peripheral oogonia; thus, a medullary/cortical gradient was observed (as previously reported (Anderson *et al.*, 2002b)). Only a limited series of experiments were performed, as this was confirmatory of previous published data (Figure 4.1).

Oogonia showed cytoplasmic staining for BDNF at all gestations examined. At earlier mid-trimester gestations, immunostaining was variable and noted to be more intense in some germ cells than others, although there was no obvious pattern or gradient identified with this. At 19 weeks, all germ cells demonstrated cytoplasmic immunostaining, with

greatest intensity surrounding the nucleus. Stromal and pre-granulosa cells also showed weak immunostaining beyond, but not before, 18 weeks gestation (Figure 4.2).

Full-length TrkB receptor protein was immunolocalised to the cytoplasm of ovarian germ cells across the gestational range examined. In particular, clear staining of the cytoplasm of oocytes in primordial follicles was observed, with faint staining of the cytoplasm of pregranulosa cells (Figure 4.1). Limited experiments attempting to localise truncated TrkB receptor protein detected no immunostaining in mid-trimester human fetal ovary.

The p75 receptor was not detected in germ cells at any gestation examined. It was, however, localized to cells of the ovarian stroma. Staining clearly demonstrated the branching pattern of the stroma from the medulla toward the surface of the ovary and surrounding clusters of germ cells. Only a limited series of experiments were performed, as this was confirmatory of previous published data (Figure 4.1).

Figure 4.1 Copy of figure showing previously published *in situ* hybridisation (A –C) and immunohistochemical localisation (D –L) of NT4, TrkB and p75 receptors in human fetal ovary (reproduced with permission (Anderson *et al.*, 2002)).

A, NT4 mRNA, 16 week ovary (antisense probe; inset shows result for sense RNA probe). **B**, NT4 mRNA, 16 week ovary at higher magnification. **C**, NT4 mRNA, 21 week ovary.

D, NT4, 13 week ovary (arrow indicates positive staining in stromal cells). **E**, NT4, 21 week ovary. **F**, NT4, 21 week ovary at higher magnification. **G**, TrkB, 18 week ovary. **H**, TrkB, 21 week ovary (inset shows same ovary at higher magnification). **I**, p75 receptor, 17 week ovary. **J**, p75 receptor, 21 week ovary. **K**, p75 receptor, 21 week ovary at higher magnification. **L**, fetal ovary, negative control omitting primary antibody.

Positive staining in all panels is brown and sections are counterstained with haematoxylin.

Oo oogonia; **o** oocyte; **pf** primordial follicle; **gc** granulosa cell; **os** ovarian stroma.

Scale bar in D and G represents 250µm and applies to H, J, L and A inset. Scale bar in E represents 1000µm and applies to I and A. Scale bar in F represents 100µm and applies to K and H inset. Scale bar in C represents 50µm and applies to B.

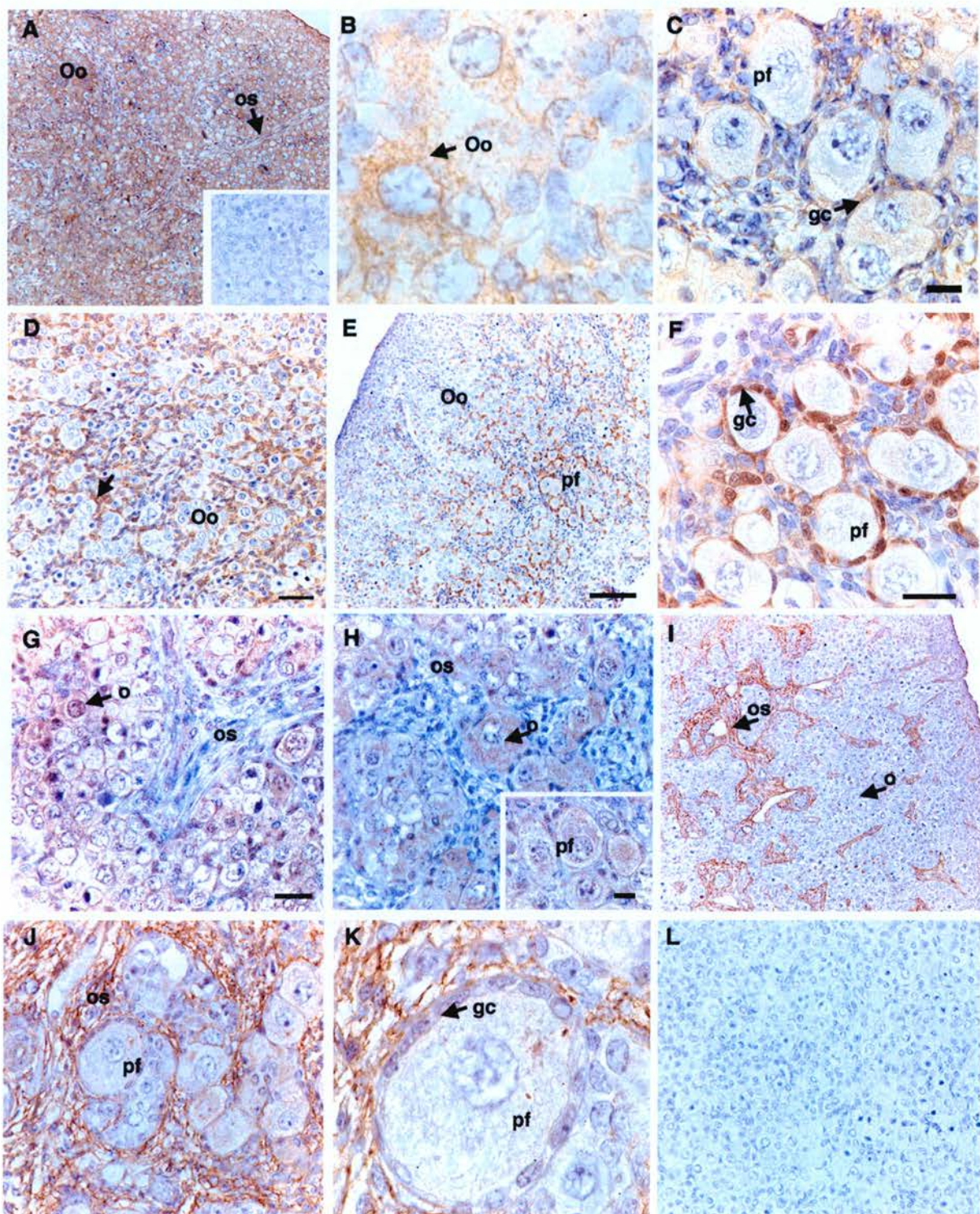


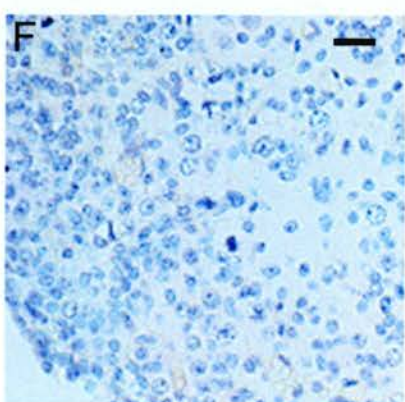
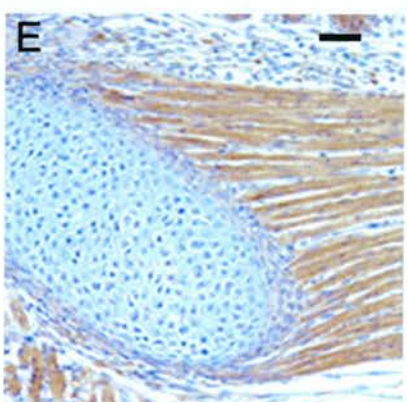
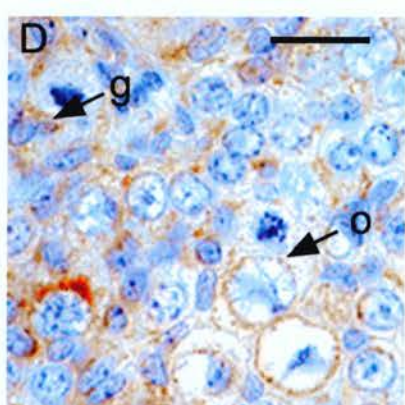
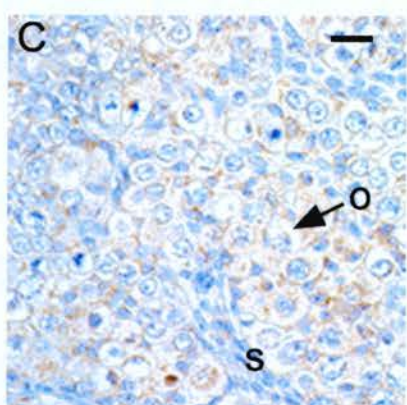
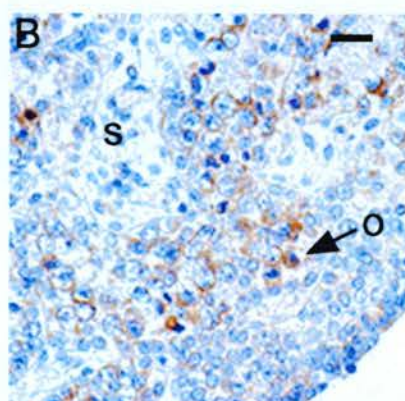
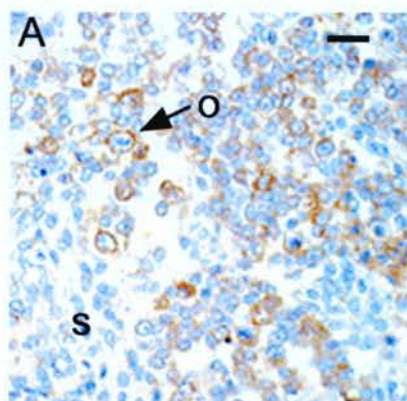
Figure 4.2 Immunohistochemical localization of BDNF in human fetal ovary.

A, 14 week ovary. **B**, 17 week ovary. **C**, 18 week ovary. **D**, 19 week ovary at higher magnification. **E**, rat brain (positive control). **F**, 14 week ovary negative control.

Positive staining in all panels is brown and sections are counterstained with haematoxylin.

s Stroma; **g** Pre-granulosa cell; **o** Oogonium.

Scale bars: 50µm in all panels.



Tissue Culture

A total of 5 separate experiments were carried out to investigate the effect of NT4 on germ cell survival and proliferation *in vitro*, using ovaries between 15 and 17 weeks gestation.

Comparison of germ cell number in time 0 and control cultures showed no significant difference between groups, demonstrating good oogonial survival in control culture conditions (31 ± 1.5 oocytes per grid after 24 hr culture v. 31 ± 1.1 in uncultured tissue).

A significant difference in total number of germ cells was seen after 24 hours culture with the various treatment conditions (ANOVA $p=0.02$). Germ cell counts following culture treatments were: 100 ng/ml NT4, 32 ± 1.6 per grid; 100 ng/ml BDNF, 32 ± 0.8 per grid; 10 μ g/ml anti-NT4, 30 ± 1.9 per grid; 100 ng/ml NT4 and 10 μ g/ml anti-NT4 combined, 31 ± 2.3 per grid; and 100 ng/ml BDNF and 10 μ g/ml anti-NT4 combined, 27 ± 1.1 per grid. Comparison of treatment with 100 ng/ml BDNF with groups treated with 100 ng/ml BDNF plus 10 μ g/ml anti-NT4 showed a mean decrease of 16% in the total number of germ cells from 32 ± 0.8 to 27 ± 1.1 per grid, $p=0.004$ (Figure 4.3A). There was also a significant difference in germ cell BrdU immunostaining (ANOVA; $p=0.001$). Results for stained germ cells counted per grid (and percentage stained out of total germ cell count) were as follows: control, 6.5 ± 1.0 ($20\% \pm 3.2$); 100 ng/ml NT4, 5.8 ± 0.6 ($18\% \pm 1.4$); 100 ng/ml BDNF, 6.0 ± 0.8 ($18\% \pm 2.2$); 10 μ g/ml anti-NT4, 6.3 ± 1.1 ($21\% \pm 4.2$); combination of 100 ng/ml NT4 and 10 μ g/ml anti-NT4, 5.6 ± 1.1

(17% \pm 3.3); and 100 ng/ml BDNF and 10 μ g/ml anti-NT4 combined, 4.6 \pm 0.6 (17% \pm 1.6). A 23% decrease in number of immunostained germ cells was seen in groups treated with 100 ng/ml BDNF and 10 μ g/ml anti-NT4, compared to 100 ng/ml BDNF (4.6 \pm 0.6 v. 6.0 \pm 0.8 per grid, $p=0.001$) (Figure 4.3B). Overall, however, there was no significant difference in proportion of germ cells immunostained, suggesting that the decreased germ cell number seen in groups treated with 100 ng/ml BDNF and 10 μ g/ml anti-NT4 was not a result of diminished proliferation but rather due to germ cell loss, either by necrosis or apoptosis (Figure 4.3C).

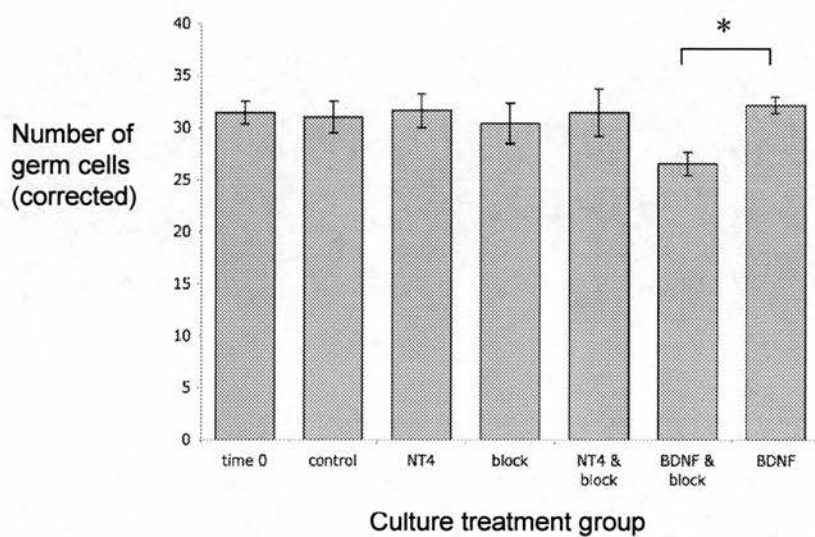
Figure 4.3 Analysis of the effect of NT4 on the number of germ cells in the human ovary.

A, Number of germ cells per grid for time 0 tissue, and various treatment groups cultured for 24 hours: control, 100ng/ml NT4, 10 μ g/ml anti-NT4 blocking antibody, both 100ng/ml NT4 and 10 μ g/ml anti-NT4, 100ng/ml BDNF and 10 μ g/ml anti-NT4, 100ng/ml BDNF alone (mean \pm SEM). * ANOVA $p=0.004$

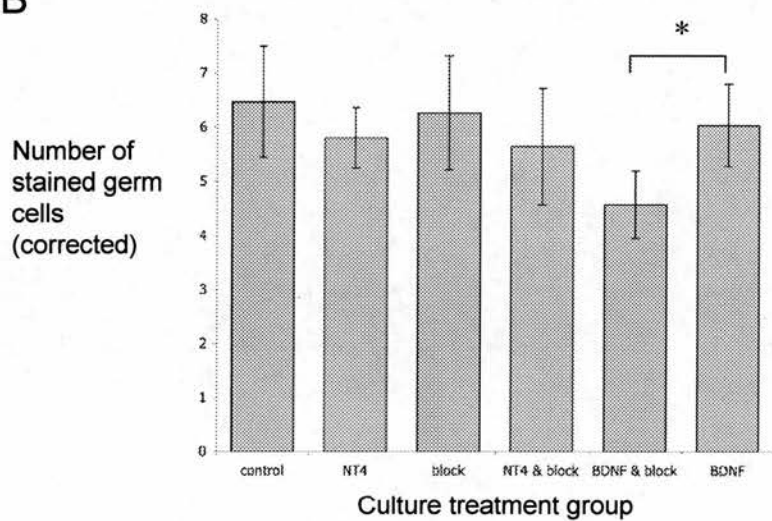
B, Number of BrdU immunopositive germ cells per grid for various treatment groups cultured for 24 hours: control, 100ng/ml NT4, 10 μ g/ml anti-NT4 blocking antibody, both 100ng/ml NT4 and 10 μ g/ml anti-NT4, 100ng/ml BDNF and 10 μ g/ml anti-NT4, 100ng/ml BDNF alone (mean \pm SEM). * ANOVA $p=0.001$

C, Percentage germ cells stained out of total germ cell count per grid for each of culture treatment groups: control, 100ng/ml NT4, 10 μ g/ml anti-NT4 blocking antibody, both 100ng/ml NT4 and 10 μ g/ml anti-NT4, 100ng/ml BDNF and 10 μ g/ml anti-NT4, 100ng/ml BDNF alone (mean \pm SEM). No significant differences detected. Similarity in percent of germ cells immunostained implies similar germ cell proliferation despite various culture conditions.

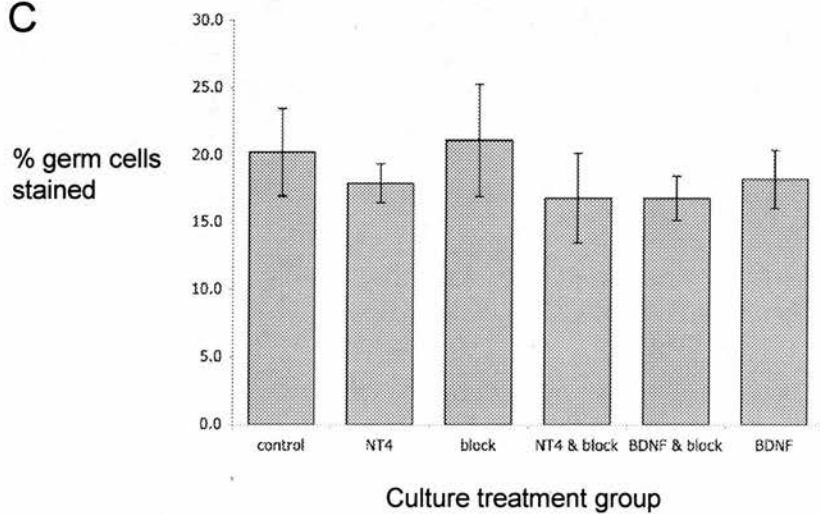
A



B



C



Significant differences were also seen in non-germ cell counts (ANOVA $p=0.02$).

Following culture treatments, non-germ cell counts were: control, 32 ± 1.5 per grid; 100 ng/ml NT4, 31 ± 1.6 per grid; 100 ng/ml BDNF, 31 ± 0.8 per grid; 10 μ g/ml anti-NT4, 33 ± 1.9 per grid; 100 ng/ml NT4 and 10 μ g/ml anti-NT4 combined, 32 ± 2.3 per grid; and 100 ng/ml BDNF and 10 μ g/ml anti-NT4 combined, 36 ± 1.1 per grid. Groups treated with 100 ng/ml BDNF and 10 μ g/ml anti-NT4 showed a 16% increase in total number of non germ-cells when compared to treatment with 100 ng/ml BDNF, increasing from 31 ± 0.8 to 36 ± 1.1 non-germ cells per grid, $p=0.001$ (Figure 4.4A).

Non-germ cell BrdU immunostaining (and percentage non-germ cells stained) was as follows: control, 2.0 ± 0.5 (7% \pm 1.7); 100 ng/ml NT4, 1.5 ± 0.3 (5% \pm 0.7); 100 ng/ml BDNF, 1.8 ± 0.3 (6% \pm 1.1); 10 μ g/ml anti-NT4, 2.2 ± 0.5 (7% \pm 1.5); combination of 100 ng/ml NT4 and 10 μ g/ml anti-NT4, 1.5 ± 0.5 (5% \pm 1.7); and 100 ng/ml BDNF and 10 μ g/ml anti-NT4 combined, 1.9 ± 0.4 (5% \pm 1.4). Using ANOVA, there was no significant difference in non-germ cell BrdU immunostaining, nor was there any significant difference in percentage of non-germ cells immunostained (Figure 4.4B-C), indicating that the reciprocal increase in non-germ cell number seen in groups treated with 100 ng/ml BDNF and 10 μ g/ml anti-NT4 reflects loss of germ cells from the tissue mass, rather than an increase in non-germ cell proliferation.

Figure 4.4 Analysis of the effect of NT4 on non-germ cells in the human ovary.

A, Number of non-germ cells per grid for time 0 tissue, and various treatment groups cultured for 24 hours: control, 100ng/ml NT4, 10µg/ml anti-NT4 blocking antibody, both 100ng/ml NT4 and 10µg/ml anti-NT4, 100ng/ml BDNF and 10µg/ml anti-NT4, 100ng/ml BDNF alone (mean ± SEM). * ANOVA $p=0.001$

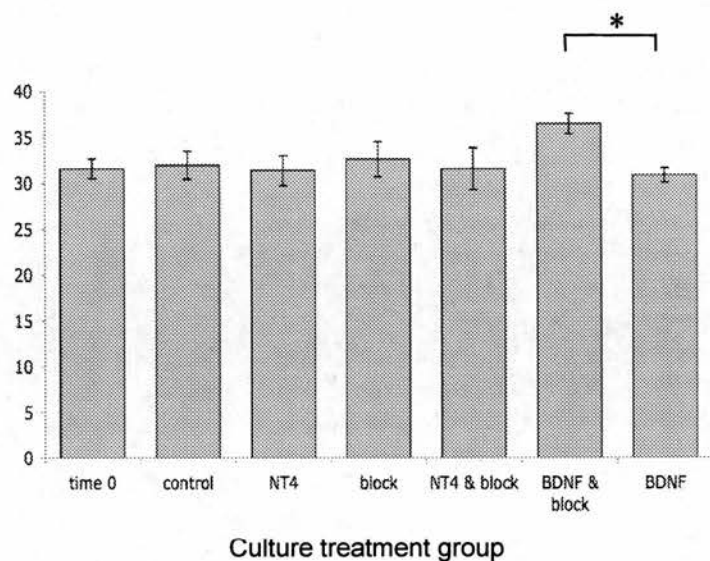
B, Number of BrdU immunopositive non-germ cells per grid for various treatment groups cultured for 24 hours: control, 100ng/ml NT4, 10µg/ml anti-NT4 blocking antibody, both 100ng/ml NT4 and 10µg/ml anti-NT4, 100ng/ml BDNF and 10µg/ml anti-NT4, 100ng/ml BDNF alone (mean ± SEM). No statistically significant differences detected.

C, Percentage germ cells stained out of total germ cell count per grid for each of culture treatment groups: control, 100ng/ml NT4, 10µg/ml anti-NT4 blocking antibody, both 100ng/ml NT4 and 10µg/ml anti-NT4, 100ng/ml BDNF and 10µg/ml anti-NT4, 100ng/ml BDNF alone (mean ± SEM). No significant differences detected.

Increase in non-germ cell number (seen after treatment with 100ng/ml BDNF and 10µg/ml anti-NT4), with no increase in BrdU staining and absence of an increase in the percent of non-germ cells immunostained, reflects germ cell loss from the tissue mass rather than non-germ cell proliferation.

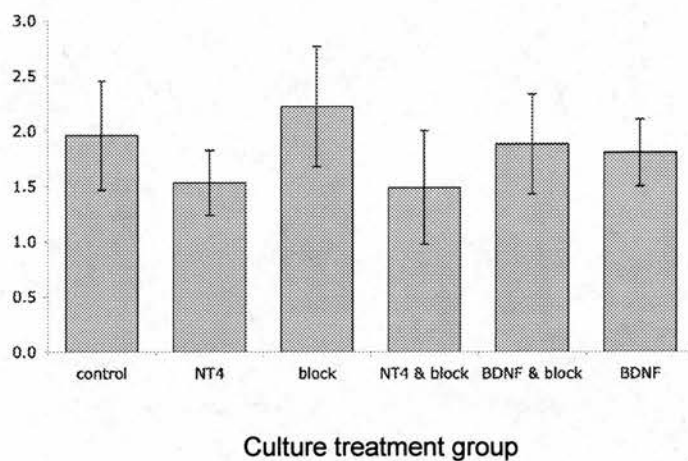
A

Number of
non-germ
cells
(corrected)



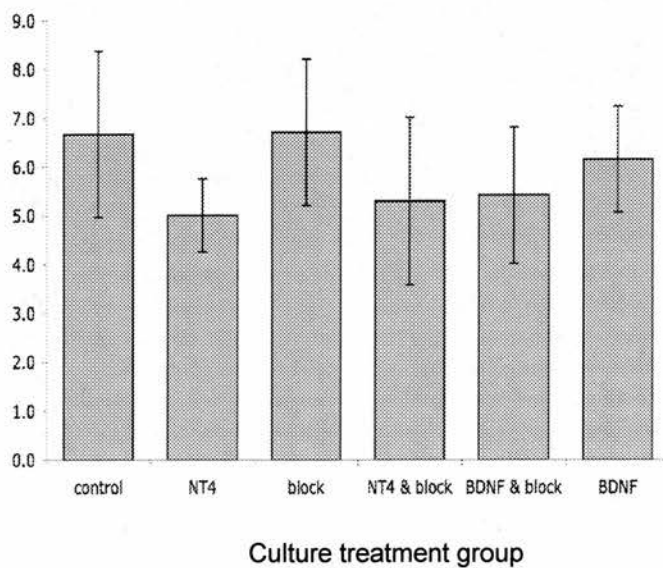
B

Number of
stained non-germ
cells
(corrected)



C

% non-germ
cells
stained



4.4 DISCUSSION

These experiments have identified expression of NT4 and BDNF ligands in the mid-trimester fetal ovary and localised them, by immunohistochemistry, predominantly to pre-granulosa and germ cell compartments respectively. Subtle evolution in immunostaining is seen with advance in gestation, concurrent with embryological development and ovarian organisation. Immunohistochemical localisation of NT4 and BDNF, as well as the TrkB receptor, supports the existence of a functional NT4 / BDNF / TrkB signalling system within the developing ovary at the time of follicular assembly.

It is now well-established that complex autocrine / paracrine interactions exist between oocytes and surrounding granulosa cells once organised as follicles. It is reasonable to assume that these interactions exist between cell types earlier in development, and are likely to be as crucial to earlier organisation and cell survival as to follicular assembly. In support of the concept of oocyte / somatic cell interaction by autocrine / paracrine communication during development, an increase in NT4, but not BDNF, mRNA occurs at the time of follicular assembly in the rodent, co-incident with appearance of TrkB mRNA (Dissen *et al.*, 1995). *In situ* hybridization studies show NT4 mRNA expression in a subpopulation of oocytes, suggesting that it may be a signalling ligand utilised by the oocyte to communicate with granulosa cells at the time of follicular formation (Dissen *et al.*, 1995) (Spears *et al.*, 2003).

Data from the culture experiments indicate neither a positive or negative effect on germ cell survival, or entry into meiosis, during mid-trimester development prior to primordial

follicle formation when culture conditions are supplemented with either NT4 or BDNF, or where the effect of endogenous NT4 is reduced by use of anti-NT4 antibody. This approach was taken because of the differential distribution of the two neurotrophins, suggesting separate physiological roles. The results demonstrate a unique feature of TrkB signalling, with a differential response to NT4 and BDNF ligands. A negative effect on germ cell survival is seen in conditions where BDNF is supplemented in the absence of NT4, suggesting that BDNF may act as a pro-apoptotic agent in germ cells in the absence of signalling by endogenous NT4. This also lends support to the theory that NT4, derived from pre-granulosa cells, is an important paracrine factor required for germ cell survival. While a differential effect of the two neurotrophins may seem surprising, there is considerable supporting evidence for this.

Structural similarities between the Trk receptor intracellular domains, including the catalytic tyrosine kinase and Shc binding site, originally led to the assumption that downstream signalling pathways would be shared. It is now understood that the various Trk receptors preferentially signal via distinct pathways (Atwal *et al.*, 2000; Yamada *et al.*, 2002), and that differential association / dissociation kinetics, competition for binding among different downstream substrates or recruitment of unique target proteins represent ways by which receptors differentially use common substrates for signalling (Qian *et al.*, 1998; Meakin *et al.*, 1999).

The p75 receptor binds all neurotrophins, but also possesses the capacity to increase ligand -Trk receptor affinity (Brennan *et al.*, 1999). Trk and p75 receptors do not bind

directly to each other, but can be complexed together (Bibel *et al.*, 1999; Lee *et al.*, 2001a). The association of the two receptor types results in a greater discrimination between neurotrophins and higher affinity ligand binding, and could potentially enable interaction of signalling pathways triggered by both receptors (Vesa *et al.*, 2000; Kanning *et al.*, 2003). BDNF, NT3 and NT4 can each bind to the TrkB receptor, but in the presence of p75 only BDNF provides a functional response (Bibel *et al.*, 1999). Signalling via the p75 receptor has been identified to mediate apoptosis in certain situations (Casaccia-Bonofil *et al.*, 1996; Bamji *et al.*, 1998; Dowling *et al.*, 1999; Roux *et al.*, 1999; Chang *et al.*, 2000) possibly by activation of the caspase cascade (Gu *et al.*, 1999a; Wang *et al.*, 2001). Apoptotic loss of neurons during periods of developmental programmed cell death carries similar analogies to germ cell loss during mammalian ovary development, and it may be that this function of p75 receptor signalling is initiated in the absence of survival trophic factors (Majdan and Miller, 1999). Indeed, BDNF causes sympathetic neuronal death by binding p75 when TrkB is absent (Bamji *et al.*, 1998). Likewise, NT4 causes p75-mediated cell death in BDNF-dependent trigeminal neurons (Agerman *et al.*, 2000). However, ovarian p75 is immunolocalised to mesenchymal cells (Anderson *et al.*, 2002b), rather than to the granulosa cells or oocytes that are subjected to apoptotic demise in the developing ovary (Hsueh *et al.*, 1994).

There are also examples of cell death mediated by Trk receptor activation. Death of medulloblastoma cell lines can be promoted by activation of Trk receptors (Muragaki *et al.*, 1997; Kim *et al.*, 1999) and necrosis of cultured cortical neurons can be provoked by

BDNF, an effect mediated by TrkB (Ishikawa *et al.*, 2000) that can be overcome by inhibiting TrkB using TrkB antisense oligonucleotides or the Trk tyrosine kinase antagonist K252a (Kim *et al.*, 2003). A similar phenomenon occurs in cultures of spinal cord neurons or pure motor neurons where exogenously provided, or endogenously produced, BDNF renders neurons vulnerable to excitotoxic insult (Fryer *et al.*, 2000; Hu and Kalb, 2003). This effect is mediated by TrkB, but not p75, receptor signalling (Hu and Kalb, 2003). Potential mechanisms by which Trk receptors could increase cellular vulnerability to death signals have thus far eluded identification, but may rest with subtle alterations in membrane electrical properties (Kalb, 2005). Another possible explanation could rest with findings from a study that demonstrates a protective effect of neurotrophins in the pathogenesis of cerebral ischaemia. Mice lacking both alleles for NT4, or deficient in a single allele for BDNF, have increased susceptibility to cerebral ischaemic damage (Endres *et al.*, 2000; Endres *et al.*, 2003). Exclusion of endogenous NT4 by the use of anti-NT4 in the culture conditions may promote germ cell susceptibility to ischaemia and thus loss by necrosis, although only when culture conditions were also supplemented with supra-physiological levels of BDNF.

Knockout mice lacking the full-length TrkB receptor cannot be used to define the role of TrkB in postnatal ovarian organisation because most animals die at birth (Klein *et al.*, 1993). A less severe phenotype is, however, observed in mice lacking both full-length and truncated receptor isoforms, as these animals can survive for up to 3 weeks after birth (Rohrer *et al.*, 1999). Ovarian follicular growth is arrested at an early (primary follicle) stage of development in the absence of both full-length and truncated TrkB

receptors, a phenotype similar to mice lacking GDF9 (Paredes *et al.*, 2004). This deficiency is accompanied by reduced granulosa cell proliferation (without obvious apoptosis) and results in disruption of crucial germ cell-somatic cell autocrine / paracrine communication processes, which are required for co-ordinated follicular growth and development. Consistent with this, transplanted *trkB*^{-/-} ovaries (from knockout mice lacking the full-length TrkB receptor) show failure of follicular development beyond the preantral stage and widespread degradation of follicular organisation due to oocyte death and an inability to support follicular growth. Loss of oocytes would suggest that a functional NT4 / BDNF / TrkB signalling complex is essential for oocyte survival during preantral follicle development.

NT4^{-/-} mice are viable and fertile, with only mild sensory deficit (Conover *et al.*, 1995; Liu *et al.*, 1995). BDNF^{-/-} mice die during early postnatal stages with severe neuronal deficits and behavioural symptoms (Ernfors *et al.*, 1994; Jones *et al.*, 1994). Knocking the NT4 gene into the BDNF locus rescues BDNF deficient mice and reveals distinct NT4 and BDNF activities, with NT4 being more effective than BDNF at promoting neuron survival and synapse formation (Fan *et al.*, 2000). Mice carrying a deletion of both NT4 and BDNF genes also show a similar deficiency of follicular growth to *trkB*^{-/-} mice. Loss of NT4 or BDNF signalling through TrkB receptors is thus likely to be the mechanism underlying the *trkB*^{-/-} phenotype, with the oocyte as the primary target of ligand-dependent receptor activation. In addition, the normal number of secondary follicles detected in mice lacking only NT4 suggests that, in the absence of this ligand, BDNF may assume a compensatory role.

In conclusion, this data has identified the importance of a balance between NT4 and BDNF signalling in determining germ cell survival during mid-trimester development prior to primordial follicle formation. These results are underpinned by the differential expression patterns of NT4 and BDNF ligands, as well as the presence of their preferential signalling receptor trkB, and support the importance of a functional NT4 / BDNF / TrkB signalling complex in the developing human ovary.

CHAPTER 5

EXPRESSION AND DISTRIBUTION OF BDNF AND RECEPTORS IN BOVINE OOCYTES AND CUMULUS AND THE EFFECT OF BDNF ON OOCYTE MATURATION

5.1 INTRODUCTION

Oocyte competence to support development after fertilisation or parthenogenetic activation is acquired gradually over the course of oogenesis and folliculogenesis and is completed during meiotic maturation (Eppig, 2001; Liu *et al.*, 2002a; Liu *et al.*, 2003). At this time the meiotic segregation of chromatin is accompanied by critical changes in oocyte cytoplasm that remain poorly defined. The significance of these changes is illustrated by the wide differences observed in embryo developmental competence following variations in oocyte *in vitro* maturation culture conditions (Keskintepe and Brackett, 1996; Krisher and Bavister, 1999; Watson *et al.*, 2000). A broad range of factors has been found to improve meiotic maturation *in vitro* and subsequent embryo developmental potential. These include supplementation of culture media with follicular fluid or serum, or specific gonadotrophins, steroid and thyroid hormones, retinoids, and different energy substrates and nutrients. These can benefit oocytes directly or via cumulus cells (reviewed by (Sutton *et al.*, 2003) (Chian *et al.*, 2004)). Specific growth factors identified as intra-ovarian regulators of oocyte maturation that have been shown to be beneficial to bovine oocyte developmental competence in *in vitro* studies, include EGF, IGF-I, activin A, inhibin A, and Midkine, a heparin-binding growth factor (Lonergan *et al.*, 1996; Stock *et al.*, 1997; Rieger *et al.*, 1998; Silva and Knight, 1998; Ikeda *et al.*, 2000).

There is increasing evidence of a role for neurotrophins in ovarian development and function, including oocyte maturation. Neurotrophins are a family of related growth factors initially identified to be important for regulation of neuronal survival and

differentiation, but which have also been described in a variety of non-neuronal tissues including the cardiovascular, immune, endocrine and reproductive systems (Yamamoto *et al.*, 1996). They include NGF, BDNF, and neurotrophins 3 and 4 (NT3 and NT4). Neurotrophins are unique in that they utilise 2 different receptors to mediate their biological actions: Trk receptors encoded by the *trk* proto-oncogene family (Trk A-C and truncated isoforms), and the p75 receptor, a member of the TNF receptor superfamily (Bibel and Barde, 2000; Rabizadeh and Bredesen, 2003). The p75 receptor is widely expressed and binds all neurotrophins. By contrast, the Trk receptors show selective affinity for different neurotrophins (i.e. TrkA for NGF, TrkB for BDNF and NT4 and TrkC for NT3). Splice variants and truncated isoforms of Trk receptors lacking intracellular tyrosine kinase domains have also been identified (Bibel and Barde, 2000)

BDNF and TrkB have been identified in the adult avian ovary (Jensen and Johnson, 2001) and NT4 expression has also been localised to the oocyte in both rodent (Dissen *et al.*, 1995) and *Xenopus* (Ibanez *et al.*, 1992). TrkB expression appears to be central to the normal formation of primordial follicles that occurs in the ovary in the few days following birth in the rodent (Spears *et al.*, 2003), and for oocyte survival during early follicular growth (Paredes *et al.*, 2004), an effect that may be predominantly mediated by truncated TrkB receptors. Mice carrying a null mutation of the NGF gene show deficient development of primordial follicles (Dissen *et al.*, 2001). A direct effect of BDNF on murine oocyte maturation in vitro has also been reported, with increased 1st polar body extrusion rate in oocytes stripped of cumulus prior to maturation (Seifer *et*

al., 2002a). BDNF is present in human follicular fluid (Seifer *et al.*, 2003), and there is evidence for increased secretion of BDNF by cumulus cells, but not mural granulosa cells, in response to cAMP (Seifer *et al.*, 2002a). These data suggest that cumulus-derived BDNF may be involved in oocyte maturation, and it is possible that its production is stimulated by gonadotrophins.

This study investigates the effects of BDNF on bovine oocyte maturation, as well as implications for embryo development after parthenogenetic activation. Parthenogenesis provides a means of assessing oocyte cytoplasmic competence to elicit development independently of sperm mediated factors, and is an accepted standard to assess oocyte viability for cloning and nuclear reprogramming (De Sousa *et al.*, 2002; Liu and Aoki, 2002). As cumulus cells and oocytes may have different capacities to respond to neurotrophin signalling, the expression and localisation of BDNF, and its receptors, is investigated.

5.2 MATERIALS AND METHODS

Tissues

Bovine ovaries were collected from a local abattoir and kept warm during transportation (Section 2.6).

Extraction of RNA and synthesis of cDNA

Total RNA was extracted from whole bovine ovary, quartered for ease of handling, and cDNA synthesis performed.

RNA was extracted from COCs, stripped oocytes and cumulus and cDNA synthesis performed. NEC control tube was used a blank for Qiagen reagents. (Section 2.8.1)

Amplification of cDNA

PCR was performed using 1 - 2 μ l cDNA samples, 5 μ M forward and reverse oligonucleotide primers and 2 x thermostart PCR mastermix in a total volume of 25 μ l (Section 2.8.2). Specific primers were used, and these and the resultant product sizes are given in Table 5.1 below. Gene specific primers were designed based on homology to Genbank sequences specified by the following accession numbers: BDNF (X97914), Trk B (NM_006180), Truncated Trk B (AF508964), p75 (AF228020). PCR amplification conditions consisted of an initial hot start denaturation step at 95°C for 15 minutes, followed by 35 - 40 cycles of denaturation at 95°C for 30 seconds, annealing at 50 - 60°C for 30 seconds and extension at 72°C for 45 seconds. A final extension period

at 72°C for 5 minutes completed the amplification. The annealing temperature varied, depending on product and primers used. The identity of all PCR products was confirmed by sequencing.

| GENE | PRIMER SEQUENCE (5' – 3') | | PRODUCT SIZE (bp) |
|-----------------|---------------------------|-------------------------|-------------------|
| BDNF | 5' | CATGGGACTCTGGAGAGCAT | 198 |
| | 3' | CAAAGGCACTTGACTGCTGA | |
| Trk B | 5' | GGCCCAGATGCTGTCATTAT | 206 |
| | 3' | TCCTGCTCAGGACAGAGGTT | |
| Truncated Trk B | 5' | CATGTTACCAATCACACGGAGTA | 430 |
| | 3' | CCATCCAGTGGGATCTTATGAAA | |
| p75 | 5' | TGGACAGCGTGACCTTCTC | 153 |
| | 3' | TCGTCTCGTCCTGGTAATAGC | |

Table 5.1. Sequences of PCR primers for BDNF and its receptors TrkB, truncated TrkB and p75.

Immunohistochemistry

Immunohistochemistry was performed on sections of whole bovine ovary and collagen-embedded COCs (Section 2.9). The conditions for individual antibodies are described below. Rat brain (donated from Dr P Saunders, MRC Human Reproductive Sciences Unit, Centre for Reproductive Health, Edinburgh) was used as positive control tissue. Immunohistochemistry method for negative controls omitted application of primary antibody in the case of p75 monoclonal antibody, substituted primary antibody with non-immune serum incubation in the case of TrkB polyclonal antibody and used a technique of pre-absorption of a blocking peptide (BDNF blocking peptide, Santa Cruz, CA, USA) in the case of BDNF.

BDNF

Non-specific binding was eliminated by pre-blocking with NSS (diluted 1:5 in TBS containing 5% bovine serum albumin). Rabbit polyclonal to BDNF (N20) (Sigma, Poole, Dorset) was used at 1:100 (incubated overnight at 4°C). Secondary swine anti-rabbit biotinylated antibody was added at 1:500.

TrkB

Non-specific binding was eliminated by pre-blocking with normal donkey serum (diluted 1:5 in TBS containing 5% bovine serum albumin). Chicken polyclonal (Promega, Southampton, UK) to TrkB was used at 1:25 (incubated overnight at 4°C). Secondary donkey anti-chicken biotinylated antibody was added at 1:500.

p75

Non-specific binding was eliminated by pre-blocking with NRS (diluted 1:5 in TBS containing 5% bovine serum albumin). Mouse monoclonal antibody to p75 (Neomarkers, CA, USA) was used at 1:25 and incubated at 4°C overnight. Rabbit anti-mouse biotinylated secondary antibody was used at a dilution of 1:500.

Oocyte Maturation *In Vitro*

Follicular aspirate cellular debris was sorted to identify and select COCs. COCs were transferred through three washes of HEPES-buffered TCM199 containing serum, then washed in serum-free base maturation medium, and randomly allocated into maturation treatment groups thereafter (15 – 25 oocytes per group). The base medium for cumulus enclosed and denuded (see below) oocyte maturation consisted of bicarbonate-buffered TCM199 supplemented with FSH, LH and oestradiol. *In vitro* maturation treatments comprised serum-free negative control, 10% FCS (positive control), 10-100 ng/ml recombinant human BDNF (PeproTech EC Ltd, London UK), 5 µg/ml monoclonal anti-human BDNF or both 10ng/ml recombinant human BDNF and 5 µg/ml monoclonal anti-human BDNF (Section 2.10).

In a second group of experiments oocytes were stripped of cumulus prior to maturation (Section 2.10). Only completely denuded oocytes were selected for maturation culture. Five cumulus-free maturation treatments were evaluated: serum-free base medium (negative control), or base medium supplemented with either 10% FCS (positive control), 10 ng/ml recombinant human BDNF, 5 µg/ml monoclonal anti-human BDNF

or both 10ng/ml recombinant human BDNF and 5 µg/ml anti-human BDNF. In each experimental replicate a sixth group of cumulus enclosed oocytes were matured in base maturation media supplemented with 10% FCS (cumulus positive control) (Section 2.10).

COCs and oocytes were cultured for 26 hours maturation and subsequently either processed for immunocytochemistry to assess meiotic progression or activated to produce parthenogenetic embryos.

Immunocytochemical analysis of meiotic progression

Maturation to MII was assessed by immunocytochemical staining of microtubules to visualise spindle morphology, of microfilaments to visualise cortical membranes and segregation of the first polar body, and by using DAPI to visualise condensed chromatin (Section 2.11). Slides were visualised using fluorescent microscopy and each oocyte scored for meiotic progression. Oocytes were scored as metaphase II arrested if they exhibited a normal alignment of chromatin along the centre of the spindle and extrusion of a single polar body containing chromatin.

Parthenogenetic Activation and Embryo Culture

Cumulus-free oocytes were parthenogenetically activated by sequential exposure to 5µM ionomycin, followed by 2.5mM DMAP and 10µg/ml cycloheximide. Oocytes

were washed following activation, then transferred into 4-well Nunc plates for final culture in 500 ml SOFaaBSA under mineral oil (Section 2.13).

Embryos were examined for cleavage at 24 hours post activation (day1), and for blastocyst development on day 7. Blastocyst cell nuclei were stained by addition of Hoescht 33342 to culture medium and embryos subsequently mounted on glass slides and visualised by fluorescence microscopy to determine their nuclear number (Section 2.14).

Statistical Analysis

The developmental data (proportion of oocytes reaching MII and blastocysts) were analysed by logistical regression. Effects of replicate and treatment and their interaction were fitted. Also, the treatment effect was split into contrasts between one or two treatments and the rest in order to examine the differences between them. Interaction between treatment and replicate was not significant. Results from all replicates were pooled for presentation. The data on blastocyst nuclei count were analysed by ANOVA to look for treatment and replicate effects.

5.3 RESULTS

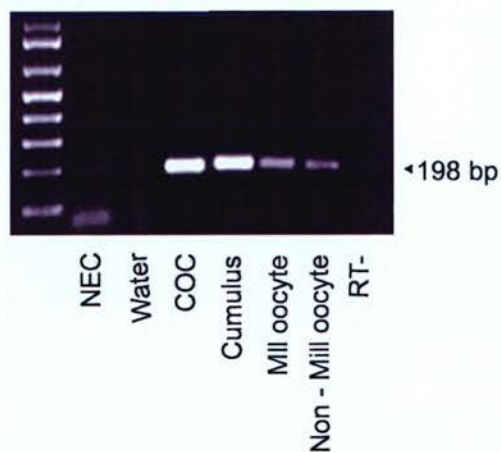
BDNF and receptor mRNA expression

Amplification products of the expected size corresponding to BDNF (198 bp), TrkB (206 bp), truncated TrkB (430 bp) and p75 (153 bp) receptors were detected in RNA extracted from bovine ovarian tissue and confirmed by DNA sequencing. All gene transcripts were also detected in RNA isolated from COCs, and cumulus cells alone (Figure 5.1). Neither full length or truncated TrkB was ever detected in oocytes in 6 independent trials, which simultaneously confirmed their presence in COCs and cumulus RNA. In contrast, both BDNF and p75 could be detected in maturing and metaphase II arrested oocytes sampled 26 h post IVM in 5 independent trials.

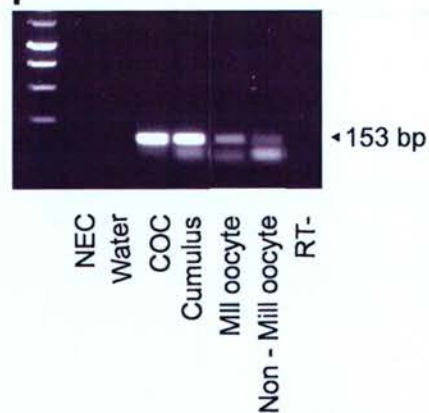
Figure 5.1 Expression of BDNF, p75, and TrkB mRNA isoforms in bovine cumulus and oocyte complexes.

RT-PCR analysis of samples prepared from biopsied bovine ovary, cumulus-oocyte complexes (COC), cumulus only or single oocytes. Oocytes collected following 26 hours IVM culture, examined for presence / absence of polar body and identified as mature (MII) or maturing (non-MII). As negative controls, PCR was performed on water, tissue / cell extract with omission of bulk first-strand cDNA synthesis (RT-) and reagent blank non-embryo control (NEC). The size of each amplicon is indicated.

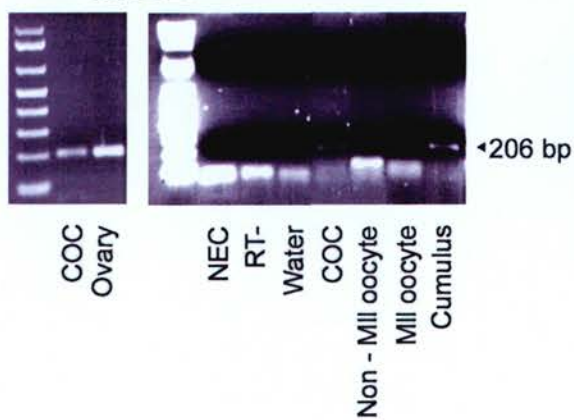
BDNF



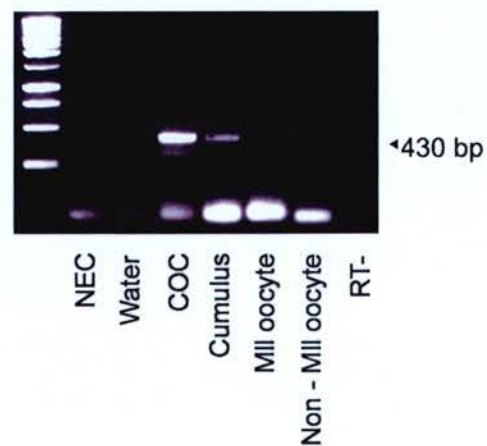
p75



TrkB



Trunc TrkB



Immunohistochemistry for BDNF and associated receptors

BDNF specific staining was evident in both oocytes and granulosa cells of preantral and antral follicles, and in expanded cumulus and oocytes following *in vitro* maturation (Figure 5.2). While BDNF expression in oocyte cytoplasm was only faint in ovarian tissue sections, it was more marked in isolated cumulus-oocyte complexes (Figure 5.2C). Cumulus cells also exhibited strong immunostaining. Some immunostaining of the theca cell layer surrounding antral follicles was also evident, as was prominent staining of vascular endothelial cells (Figure 5.2B). No staining was detected in negative controls, following preabsorption of the primary antibody with BDNF peptide (Figure 5.2 D-F).

Similar to BDNF, the p75 receptor was expressed by granulosa cells of preantral and antral follicles (Figure 5.2 G-H), with faint staining of theca cells surrounding antral follicles (Figure 5.2H). Oocytes of isolated cumulus-oocyte complexes were positive for p75-specific immunohistochemical staining (Figure 5.2I).

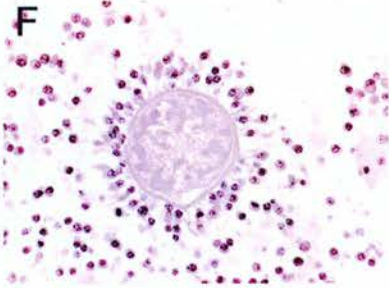
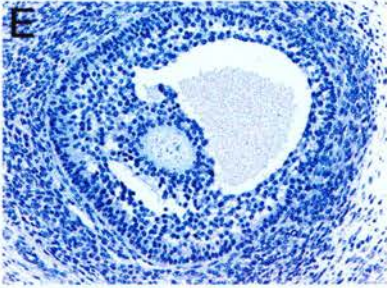
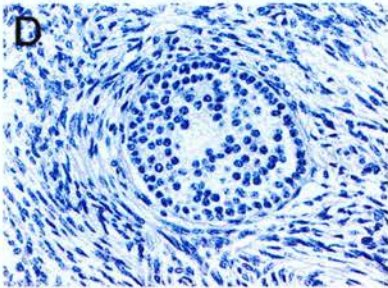
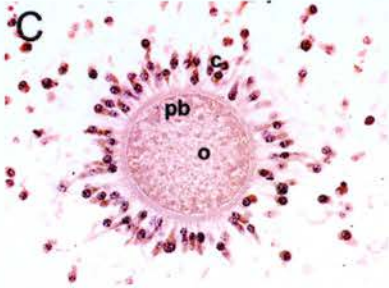
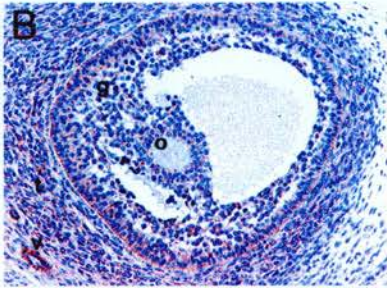
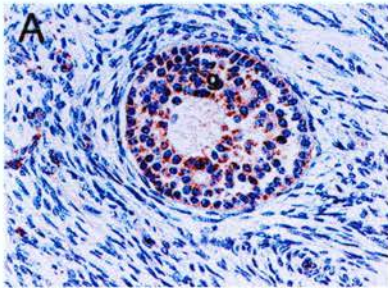
We were unable to obtain clear specific immunostaining for TrkB, although staining was detected in rat brain as a positive control (not shown).

Figure 5.2. Immunohistochemical staining for BDNF and p75 in bovine ovarian follicles and cumulus oocyte complexes.

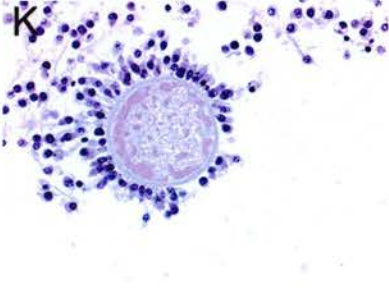
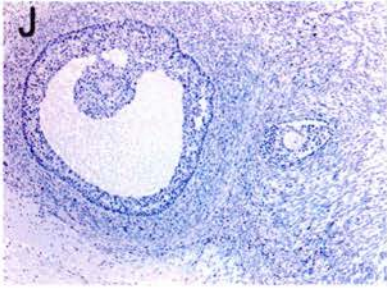
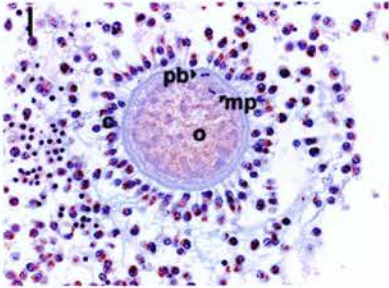
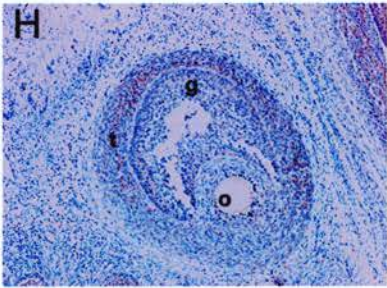
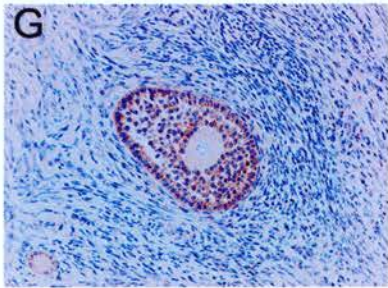
BDNF specific immunostaining was evident in both oocytes (**o**) and granulosa (**g**) cells of preantral (**A**) and antral (**B**) follicles and in cumulus (**c**) and oocytes following in vitro maturation of isolated cumulus-oocyte-complexes (**C**). Some immunostaining of the theca (**t**) cell layer surrounding antral follicles was also evident, as was prominent staining of vascular (**v**) endothelial cells. In matched serial sections, this staining was blocked (**D-F**) if the primary antibody was first pre-incubated with the BDNF blocking peptide.

Immunostaining for p75 (**G-I**) which was above that observed following omission of the primary antibody (**J, H**), was also detected in the granulosa and thecal cell layer of preantral (**G**) and antral follicles (**H**), and in oocytes and cumulus cells of in vitro matured cumulus-oocyte complexes (**I**). Polar bodies (**pb**) and a metaphase plate (**mp**) are labelled.

BDNF



p75



Effect of BDNF on maturation of cumulus-enclosed oocytes to MII

To evaluate the effect of BDNF on bovine oocyte maturation, cumulus-enclosed oocytes were matured in the presence (positive control) or absence (negative control) of 10% FCS, or in serum-free media supplemented with 10ng/ml or 100ng/ml BDNF, 5µg/ml anti-BDNF blocking antibody, or both 10ng/ml BDNF and 5µg/ml anti-BDNF blocking antibody. All oocytes in each treatment group had undergone GVBD by the time they were analysed.

From reviewing tabulated data, it was clear that success rate for COCs reaching MII varied between replicate experiments. It was anticipated that COCs matured with FCS would show highest MII rates and this was taken into account when choosing the method of analysis. Logistic regression models were fitted to test for differences between treatments, the difference between FCS and other the treatments, differences between replicates and a possible interaction between treatment effect and replicate. No evidence of an interaction was found. The differences between replicates was significant, but the success rates for all treatments were found to vary over replicates; there was no change to the relative success rates between treatments. In total 77% of COCs in maturation medium containing FCS were scored as MII arrested after 26 h of culture. This was 2-3 fold greater ($p<0.01$) than that observed in all other treatment groups. No significant difference in outcome was found between any other treatment group (Figure 5.3). Thus, supplementation of serum-free maturation medium with BDNF was insufficient to substitute for the known benefit of serum to support nuclear maturation, when assessed at a point in time when bovine oocytes should have reached MII. Furthermore, a

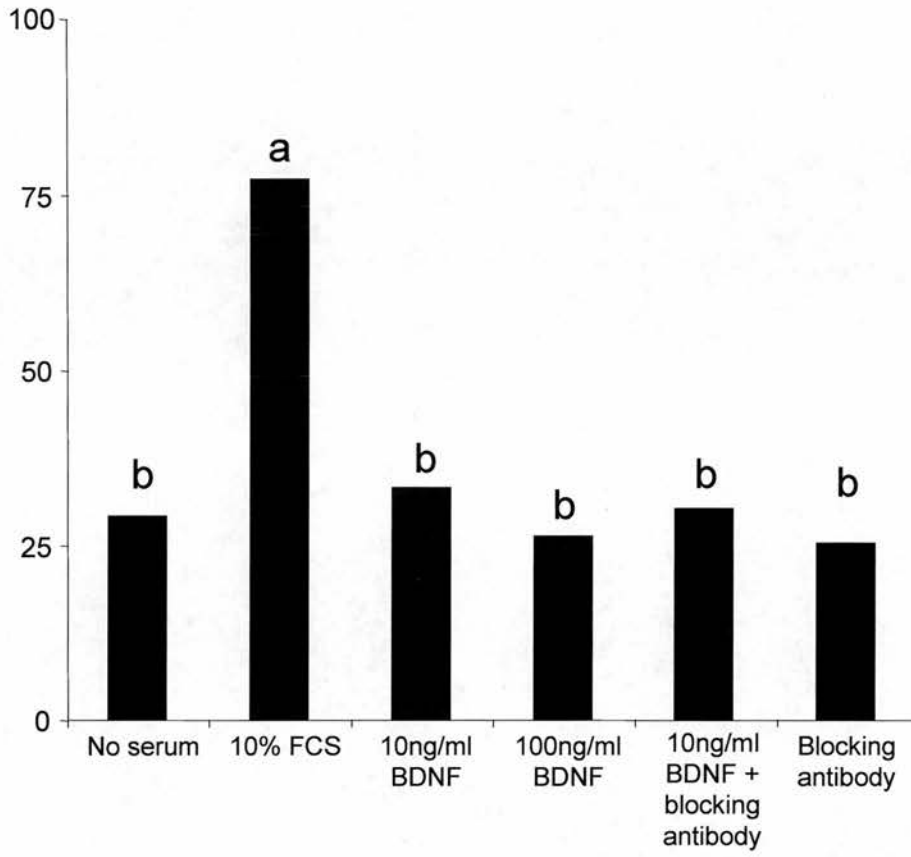
blocking antibody to BDNF did not have any additive detrimental effect on nuclear maturation above that observed for serum-free maturation in general.

Figure 5.3. Effect of BDNF on maturation of cumulus-enclosed oocytes to MII.

COCs were matured in the presence (positive control) or absence (negative control) of 10% FCS, or in serum-free media supplemented with 10ng/ml or 100ng/ml BDNF, 5µg/ml anti-BDNF blocking antibody, or 10ng/ml BDNF and 5µg/ml anti-BDNF. The experiment was repeated 4 times to arrive at the average proportion of oocytes at MII after 26h of culture. Number of COC per group: serum-free n=49; 10% FCS n=43; 10ng/ml BDNF, n=49; 100ng/ml BDNF, n=47; 5µg/ml anti-BDNF, n=48; 10ng/ml BDNF and 5µg/ml anti-BDNF, n=47.

Significantly higher proportion MII found in COCs cultured with FCS (denoted a) versus all other groups (denoted b; $p < 0.01$).

% COCs at MII after in vitro maturation



Effect of BDNF during maturation of cumulus enclosed oocytes on parthenogenetic embryo development

In the next two experimental series parthenogenetic embryo development to cleavage and blastocyst stages was evaluated following activation of oocytes matured as COCs in the previously defined treatment groups. In these trials, no attempt was made to score maturation status of oocytes following *in vitro* maturation, thus most oocytes except those damaged during removal of cumulus subsequently underwent parthenogenetic activation, irrespective of maturation status. In a first series consisting of three replicate trials, COCs matured without serum and activated (total n= 83) were compared with those matured with FCS (n=103) or either 10ng/ml (n=95) or 100 ng/ml BDNF (n=91; Figure 5.4 A-C). The proportion of activated oocytes undergoing first cleavage after 24 hours showed no significant differences across treatment groups (serum-free 63%; FCS 65%; 10ng/ml BDNF 65%; 100ng/ml BDNF 74%; Figure 5.4A). By contrast, the proportion of activated oocytes forming blastocysts following maturation with 10ng/ml BDNF was over 1.5 fold higher ($p<0.01$) than with FCS, which was equivalent to the higher concentration of BDNF. Blastocyst yields in all treatment groups were also significantly higher than oocytes matured without serum (serum-free 10%; FCS 15%; 10ng/ml BDNF 24%; 100ng/ml BDNF 18%; $p<0.01$; Figure 5.4B). Improved blastocyst development following maturation with the lower concentration of BDNF was also apparent if calculated relative to the number of cleaved embryos (serum-free 15%; FCS 22%; 10ng/ml BDNF 37%; 100ng/ml BDNF 24%; $p<0.01$; Figure 5.4C).

Figure 5.4 Development of parthenogenetic embryos following *in vitro* maturation of cumulus-enclosed oocytes with BDNF.

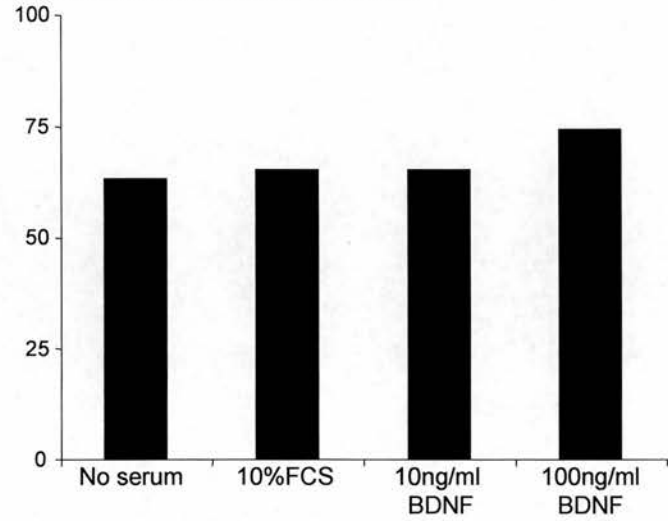
COCs were matured in the presence (positive control) or absence (negative control) of 10% FCS, or in serum-free media supplemented with 10ng/ml or 100ng/ml BDNF then activated. Data from three replicate trials were pooled. Number of activated oocytes per group: 10% FCS, 103; serum-free, 83; 10ng/ml BDNF, 95; 100ng/ml BDNF, 91.

A, Proportion of activated eggs undergoing cleavage. No significant difference detected between treatment groups.

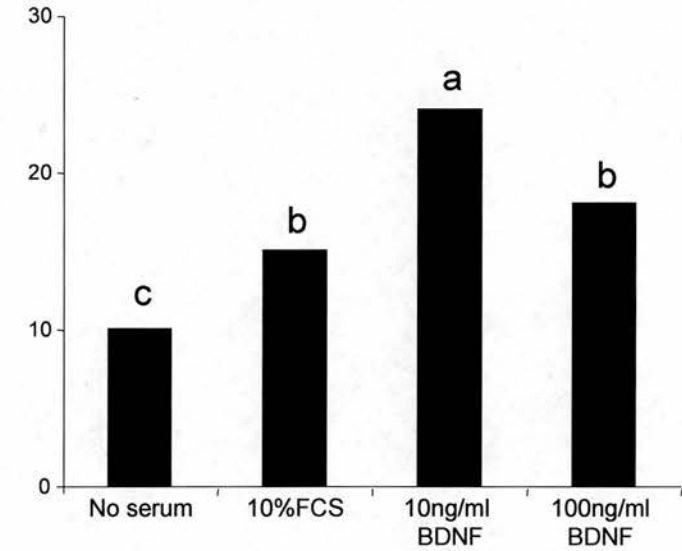
B, Proportion of activated eggs forming blastocysts. Statistically significant increased number blastocysts achieved after maturation with 10ng/ml BDNF versus all other groups (a; $p < 0.01$) COCs matured with serum or 100ng/ml BDNF were comparable (denoted b), but achieved statistically significantly higher proportion of blastocysts than maturation in absence of serum (denoted c; $p < 0.01$).

C, Proportion of cleaved embryos forming blastocysts. Significant differences also apparent where blastocyst development calculated relative to number of cleaved embryos. As before, different superscripts denote significant differences ($p < 0.01$)

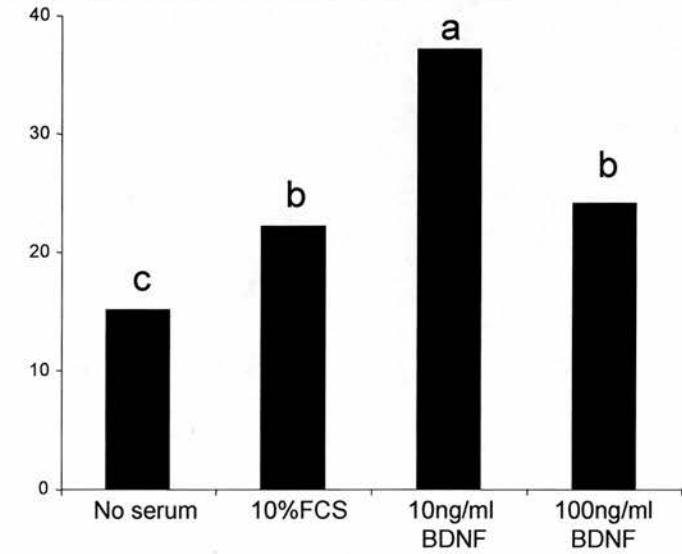
A % Oocytes undergoing cleavage



B % Oocytes forming blastocysts



C % Cleaved embryos forming blastocysts



In a second series of three replicate trials, the development of COCs matured without serum was compared against those matured with 10ng/ml BDNF (the best treatment in the previous series), 10ng/ml BDNF and 5µg/ml anti-BDNF blocking antibody, or 5µg/ml anti-BDNF alone. The proportion of activated oocytes undergoing first cleavage after 24 h was significantly lower following maturation with the blocking antibody alone (30%, n=81) versus all of the other treatment groups (serum-free 64%, n=83; 10ng/ml BDNF 60%, n=85; 10ng/ml BDNF and 5µg/ml anti-BDNF 63%, n=76; $p<0.01$).

Blastocyst yields in this experimental series were generally poorer than that observed in the first series, possibly due seasonal variations in oocyte quality commonly encountered with abattoir derived ovaries. Despite this, treatment with 10ng/ml BDNF yielded significantly more blastocysts per activated oocytes compared to any of the other treatment groups (10ng/ml BDNF 9%; serum-free 2%; 10ng/ml BDNF and 5µg/ml anti-BDNF 4%; anti-BDNF alone 4%; $p<0.01$). Evaluating blastocyst nuclear counts for each treatment within and between experiment trials revealed no significant differences. For each treatment group, mean \pm S.E.M: serum-free 68 ± 8 , n=12; FCS 68 ± 5 , n=15; 10ng/ml BDNF 65 ± 6 , n=31; 100ng/ml BDNF 77 ± 6 , n=16; 10ng/ml BDNF and 5µg/ml anti-BDNF 54 ± 6 , n=3; 5µg/ml anti-BDNF alone 63 ± 10 , n=3.

Effect of BDNF on maturation of cumulus-free oocytes to MII

To evaluate the effect of BDNF on bovine oocyte maturation without cumulus, germinal vesicle oocytes were denuded at the time of collection and matured in medium with or without 10% FCS, or in serum-free media supplemented with 10ng/ml BDNF, 10ng/ml BDNF and 5µg/ml anti-BDNF, or 5µg/ml anti-BDNF antibody alone. These were compared against cumulus enclosed oocytes matured with 10% FCS (positive control). At the time of analysis all oocytes had undergone GVBD. It was anticipated that COC and denuded oocytes matured with FCS would be the most successful treatments and the overall tabulated results confirmed this. The same analysis was used as before, except that the COC and FCS treatments were considered as potentially different from the rest. There was no significant interaction between treatment and replicate and no significant difference between replicates. Similar to the first maturation experiment evaluating BDNF on cumulus enclosed oocytes, 71% (n=73) of COCs matured with FCS were scored as MII arrested after 26 h of culture. This was significantly higher than cumulus-free oocytes matured with FCS (39%, n=69; $p<0.01$), which in turn was approximately double ($p<0.01$) that observed in all of the other treatment groups (serum-free 22%, n=69; 10ng/ml BDNF 25%, n=71; 10ng/ml BDNF and 5µg/ml anti-BDNF 19%, n=36; and 5µg/ml anti-BDNF alone 18%, n=49). Thus, although progression to MII is generally poorer in the absence of cumulus cells, exogenous BDNF cannot substitute for serum to support nuclear maturation in the presence or absence of cumulus cells. Furthermore, a blocking antibody to BDNF did not have any additive detrimental effect on nuclear maturation beyond that observed for serum-free maturation in general.

Effect of BDNF during maturation of cumulus free oocytes on parthenogenetic embryo development

In a final series of experiments, parthenogenetic embryo development to cleavage and blastocyst stages was evaluated following activation of oocytes matured in the absence of cumulus cells as described above. As with the activation experiments involving cumulus-enclosed maturation, the maturation status of oocytes was not scored and all morphologically intact oocytes were activated. This design was repeated in 5 replicate trials, each of which also included a positive control of COCs matured with 10% FCS (Figure 5.5 A-C). The proportion of activated oocytes that had cleaved by 24 h was significantly higher for COCs and stripped oocytes matured with FCS and stripped oocytes matured with BDNF, compared to any other treatment group (COCs (serum) 61%, n=114; stripped 10% FCS 53%, n=119; stripped serum-free 32%, n=115; 10ng/ml BDNF 43%, n=116; 10ng/ml BDNF and 5µg/ml anti-BDNF 29%, n=118; 5µg/ml anti-BDNF antibody alone 28%, n=120; $p<0.05$). Similarly, the proportion of activated oocytes forming blastocysts was significantly highest for COCs matured with serum (33%). Cumulus-free oocytes matured with serum (13%) or 10ng/ml BDNF (7%) were in turn significantly different from BDNF with blocking antibody (4%), and serum-free (2%), with no blastocysts formed by treatment with blocking antibody alone ($p<0.01$). A similar relationship between treatment groups was observed if blastocyst yields were expressed relative to numbers of cleaved embryos ($p<0.01$), with the exception that there no longer was a significant difference between BDNF and BDNF with blocking antibody. Irrespective of differences in the proportion of embryos forming blastocysts

there were no differences in blastocyst nuclear counts between cumulus-free treatment groups (mean \pm S.E.M, stripped 10% FCS 61 ± 4 , $n=15$; stripped serum-free 53 ± 13 , $n=2$; 10ng/ml BDNF 57 ± 6 , $n=8$; 10ng/ml BDNF and 5 μ g/ml anti-BDNF 59 ± 5 , $n=5$). However, blastocyst nuclear counts for COCs matured in medium containing serum were significantly higher than cumulus-free groups (85 ± 8 , $n=38$; $p<0.01$). These findings suggest that BDNF can substitute for serum but not cumulus cells during IVM in order to support oocyte competence to form parthenogenetic blastocysts. The capacity of IVM treatment with blocking antibody to completely block blastocyst formation suggests that BDNF may also exert an influence on oocytes directly.

Figure 5.5 Development of parthenogenetic embryos following *in vitro* maturation of cumulus-free oocytes with BDNF or blocking antibody.

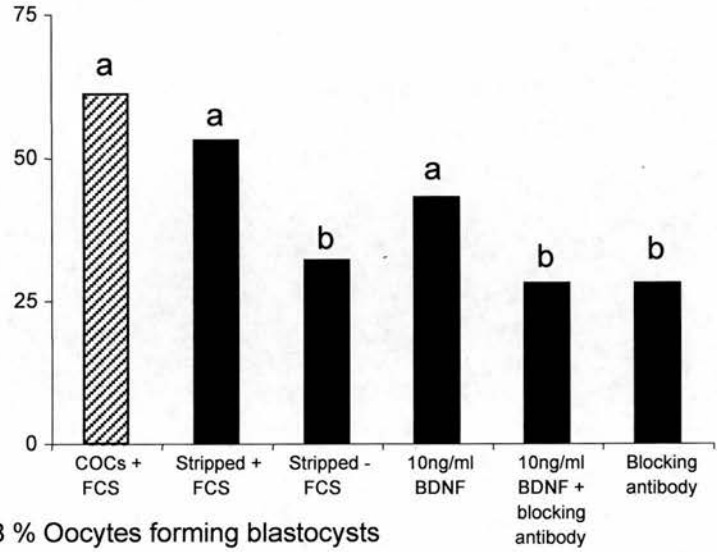
Oocytes were denuded at the time of collection and matured in medium with or without 10% FCS (serum), or in serum-free media supplemented with 10ng/ml BDNF, 10ng/ml BDNF and 5µg/ml anti-BDNF blocking antibody, or 5µg/ml anti-BDNF antibody alone prior to activation. Data from five replicate trials were pooled. Number of activated oocytes per group: COCs 10% FCS, 114; stripped oocytes 10% FCS, 119; stripped oocytes serum-free, 115; 10ng/ml BDNF, 116; 10ng/ml BDNF and 5µg/ml anti-BDNF, 118; 5µg/ml anti-BDNF alone, 120.

A, Proportion of activated eggs undergoing cleavage. Significant differences detected between COC, FCS and 10ng/ml BDNF treatments (denoted a) versus other groups (denoted b). $p < 0.05$.

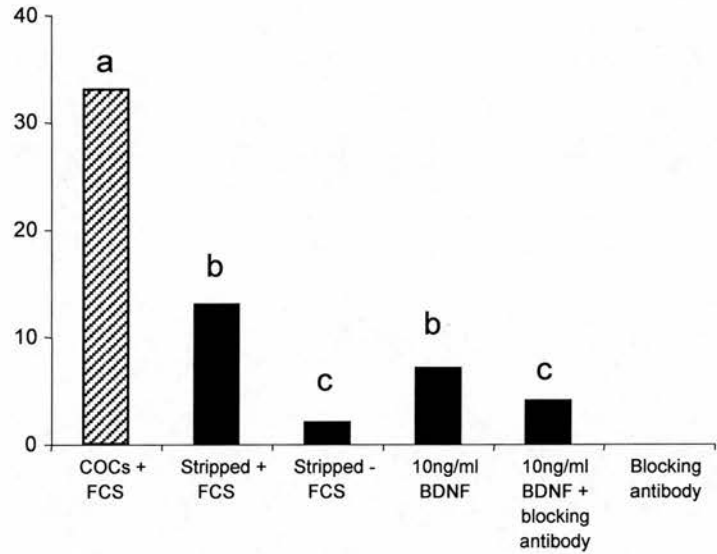
B, Proportion of activated eggs forming blastocysts. COC maturation yields highest number of blastocysts versus all other groups (a; $p < 0.01$). Stripped oocytes matured with FCS or 10ng/ml BDNF (denoted b) show statistically significant better results than stripped oocytes matured without serum or with 10ng/ml BDNF plus blocking antibody (denoted c; $p < 0.01$). No statistical significant differences between FCS and 10ng/ml BDNF, or between no serum and 10ng/ml BDNF plus blocking antibody.

C, Proportion of cleaved embryos forming blastocysts. Significant differences denoted by different superscripts as previously ($p < 0.01$).

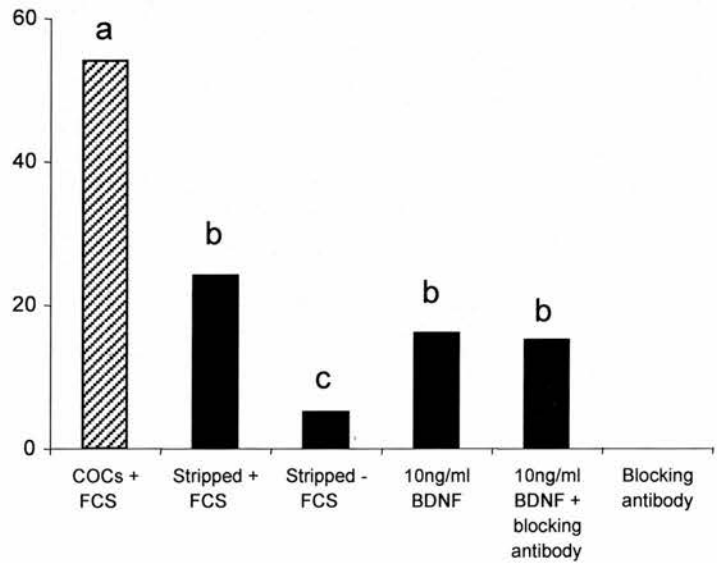
A % Oocytes undergoing cleavage



B % Oocytes forming blastocysts



C % Cleaved embryos forming blastocysts



5.4 DISCUSSION

A bovine oocyte *in vitro* maturation and parthenogenesis model was used to investigate the role of BDNF in conferring oocyte developmental competence. Both oocytes and cumulus express mRNA transcripts and protein for BDNF and the p75 receptor that has affinity for all neurotrophins, but transcripts for the full length and truncated forms of the specific TrkB receptor are confined to cumulus. BDNF cannot substitute for serum in supporting meiotic progression to MII during *in vitro* maturation of cumulus enclosed or free oocytes. Despite this it can increase or approach the yield of parthenogenetic blastocysts relative to serum mediated maturation of cumulus enclosed or free oocytes, respectively, via signalling pathways that are sensitive to a BDNF specific blocking antibody. These results suggest that BDNF plays a role in conferring oocyte cytoplasmic competence to support early embryo development, independently of nuclear maturation, and that this may involve both autocrine and paracrine signalling within cumulus oocyte complexes.

This study relied on parthenogenesis rather than fertilisation to assess oocyte competence for embryonic development. In mammals parthenogenesis can yield viable offspring provided that parent-specific imprints regulating gene expression are overcome to permit the formation of a functional placenta (Kono, 2006). Without such manipulations, parthenogenesis still provides a valuable measure of oocyte competence to initiate a developmental program since development to the blastocyst stage is independent of epigenetic imprinting (Latham *et al.*, 1994). Accordingly, it is commonly used to assess oocyte competence to support early development following somatic or

pronuclear transfer (Liu *et al.*, 2001) (De Sousa *et al.*, 2002). The method of parthenogenetic activation used in our study has previously been characterised to yield equivalent rates of development to the blastocyst stage in a direct comparison with fertilised oocyte cohorts, although with a significant reduction in resulting cell number, similar to the present results (De La Fuente and King, 1998).

Important roles for Trk B receptors in oocyte survival in both the perinatal period of primordial follicle formation and during the early stages of follicle growth have been demonstrated using mouse knockout models (Spears *et al.*, 2003) (Paredes *et al.*, 2004). Unfortunately these models fail to illuminate the roles for these receptors or BDNF in the later stages of ovarian follicle development and for oocyte competence, due to the neonatal lethality that they invoke (Klein *et al.*, 1993; Ernfors *et al.*, 1994). BDNF, NT-4 and NT-3 have all been detected in adult human follicular fluid (Seifer *et al.*, 2002b; Seifer *et al.*, 2003). *In vitro* studies on human cumulus cells have also found that BDNF secretion can be stimulated by treatment with cAMP and human chorionic gonadotropin (hCG), but not recombinant FSH (Feng *et al.*, 2003). Both BDNF and NT-4, but not NT-3 also promoted 1st polar body extrusion in cumulus-free mouse oocytes matured *in vitro*, compared with those matured in the absence of serum or hormones (Seifer *et al.*, 2002b; Seifer *et al.*, 2002a). These publications report that most mouse oocytes are immunopositive for TrkB, the BDNF and NT-4 receptor tyrosine kinase, but not TrkC, the respective receptor for NT-3. The RT-PCR data in this study identify p75, but not TrkB isoform mRNAs, in bovine oocytes. Furthermore, there were no significant differences between BDNF and serum-free treatment groups in the proportion of oocytes

reaching MII, both of which were inferior to supplementation with serum. This may reflect a species difference.

Our current study provides the first evidence that BDNF may benefit oocyte competence for embryonic development without necessarily benefiting maturation. Whereas approximately 80% of oocytes matured with FCS (positive control) reached MII, this was apparently reduced to 30 % in all FCS-free experimental treatments, including those with BDNF. However, in succeeding trials there was no difference between supplementation with BDNF and FCS in the proportion of parthenogenetic embryos that cleaved (~60%), with blastocyst yields improved or matched by BDNF treatment. Although serum-free IVM has previously been shown to result in a significant reduction in the proportion of oocytes reaching MII, our yield in the absence of serum was still lower than the 50-60% reported by others (Lonergan *et al.*, 1994a; Ali and Sirard, 2002). However, our observations were consistent across replicate trials, with FCS supplemented cohort oocytes providing expected MII yields. The fact that in subsequent trials there was no difference in parthenogenetic embryo cleavage between FCS containing and free IVM treatments suggests that our immunocytochemical method for evaluating MII may have underestimated this event. This presumes however, that parthenogenetic embryo cleavage is linked to MII in the same way that fertilised embryo cleavage is. This is unknown but not necessarily the case, considering differences between the two situations in signal transduction and resulting spindle dynamics (Navara *et al.*, 1994). Cumulatively, our results substantiate a role for BDNF in enabling both early cleavage development, driven by maternal factors in the oocyte, and blastocyst

formation, requiring the activation and maintenance of zygotic transcription. For both cumulus enclosed and free maturation, the effect of the BDNF blocking antibody was first manifest by a significant reduction in cleavage. However, compared to the serum treatment group, the improvement or matching of blastocyst yields with exogenously supplied BDNF was not paralleled by improved cleavage. Since BDNF did not increase the quantity of cells in blastocysts, it is likely that its effect was on oocyte and embryo survival as opposed to the promotion of growth.

BDNF signalling between cumulus and oocytes may be bi-directional with functionally different consequences given that both cell types express BDNF and differentially express p75 and TrkB isoforms, namely oocytes lack the latter. Trk and p75 receptors do not bind directly to each other, but can be complexed together (Bibel *et al.*, 1999; Lee *et al.*, 2001a). This allows the signalling pathways triggered by both receptors to interact. The association of the two receptor types apparently results in higher affinity ligand binding and a greater discrimination between neurotrophins. Thus, BDNF, NT3 and NT4 can each bind to the TrkB receptor, but in the presence of p75 only BDNF provides a functional response (Bibel *et al.*, 1999). In neurons, Trk receptors and their substrates can activate three main signalling cascades: 1) Differentiation, via a Ras/Raf/MEK/MAP kinase pathway, 2) cell survival (anti-apoptosis), by association with insulin receptor substrates leading to inactivation of proapoptotic proteins, and 3) calcium release from internal stores via PLC- γ mediated production of IP3 and production of protein kinase C, which in neurons plays a role in neurotrophin mediated neurotrophin

release (Bibel and Barde, 2000). All of these pathways may be relevant to follicular and oocyte maturation, but most striking is the potential for neurotrophin mediated stimulation of the MAP kinase pathway. Gonadotropin induced cumulus expansion and resumption of meiosis in oocytes is dependent upon activation of MAP kinase in granulosa cells. This activation is downstream of gonadotropin-induced elevation of granulosa cell cAMP, and is dependent upon one or more paracrine factors from the oocyte (Su *et al.*, 2003). BDNF secretion by cumulus is another consequence of either gonadotropin stimulation or artificial elevation of cAMP. Elevated cAMP in cumulus could lead to the same in oocytes via gap junctions, culminating in BDNF secretion by oocytes that could act in an autocrine or paracrine fashion to augment the effect of cumulus-derived BDNF. It is unknown whether BDNF derived from oocytes or cumulus would differ in their bioactivity. Neurotrophins in general are substrates for pro-protein convertases that can alter the molecular bioactivity of their targets by proteolytic cleavage during intracellular and extracellular processing. BDNF is an especially well characterised example of convertase-dependent modulation of bioactivity (Mowla *et al.*, 1999). Thus, differences in BDNF bioactivity could be achieved by differential expression of pro-protein convertases in cumulus and oocytes.

The functional consequences of ligand interaction with the p75 receptor are also complex, and in neurons have been linked to cell survival, arrest, differentiation, and programmed cell death. Recently the concept of p75 as a 'quality control' receptor has been advanced based on evidence that it is capable of mediating programmed cell death in response to either ligand binding or withdrawal. In essence the downstream

consequence of ligand interactions are proposed to be dependent on the relative proportions of p75 and Trk receptors and which neurotrophin is present (Rabizadeh and Bredesen, 2003). According to this model, binding of neurotrophins to p75 in the absence of Trk receptors, typically suppresses apoptosis. This could be the case for bovine oocytes provided they do not express TrkA or C receptors. From mouse knockout models, mice homozygous for a targeted mutation in p75, rendering it functionally inactive, are viable and fertile, although they eventually develop deficits in their peripheral sensory nerves characterised by heat sensitivity and susceptibility to ulceration (Ernfors *et al.*, 1994). This suggests that the most critical role for p75 may be to ameliorate the effect of stress, which in the case of oocyte maturation and developmental competence might be most affected by adverse *in vitro* culture environments.

A number of other growth factors have been reported to improve oocyte competence and to support embryo development, when applied during oocyte maturation *in vitro*. EGF improves the yield of fertilised bovine blastocysts, but not cell number, relative to serum-free culture conditions lacking hormones (Lonergan *et al.*, 1996). However, this effect is preceded by promotion of the proportion of cumulus enclosed or free oocytes progressing to MII, the latter supporting a direct effect on oocytes. EGF-receptor tyrosine kinase mediated activation of meiosis was first described in *Xenopus* oocytes, and later confirmed in the rat and mouse (Maller, 1985; Ueno *et al.*, 1988) (Downs, 1989). Studies in the latter also revealed a direct effect of EGF on cumulus leading to the promotion of cumulus expansion. Like EGF, other groups have demonstrated that

supplementation of bovine oocyte *in vitro* maturation culture with exogenous activin A promotes meiotic maturation and subsequent yield of fertilised blastocysts (Stock *et al.*, 1997; Silva and Knight, 1998), an effect which is reduced by the activin binding protein follistatin. These gene products are synthesised by granulosa cells and are prominent components of follicular fluid (Braw-Tal *et al.*, 1994). Activin A receptor mRNA can be detected in both granulosa cells and oocytes (Cameron *et al.*, 1994). In the former, activin A appears to increase the number of FSH receptors in granulosa cells and as such may promote gonadotropin action during maturation (Nakamura *et al.*, 1993). A direct effect of activin A on oocytes is supported by evidence of improved progression to MII of cumulus-free oocytes, although the mechanism by which this is achieved is unknown (Stock *et al.*, 1997). Activin A also promotes human germ cell survival and proliferation prior to primordial follicle formation, as discussed earlier in Chapter 3 (Martins da Silva *et al.*, 2004). Only midkine, a heparin-binding growth/differentiation factor, which is also a prominent feature of bovine follicular fluid, is similar to BDNF in promoting oocyte competence to reach the blastocyst stage without also improving meiotic progression to MII (Ikeda *et al.*, 2000). Midkine is also produced by granulosa cells under the control of gonadotropins (Minegishi *et al.*, 1996). Unlike BDNF however, midkine does not appear to act directly on oocytes. Its actions can also be blocked by heparin (Ikeda *et al.*, 2000).

In conclusion, this study adds BDNF to the list of growth factors normally present in follicular fluid, which can specifically promote oocyte developmental competence during maturation *in vitro*. However, BDNF differs from most other factors identified in

that its effect is to promote cytoplasmic maturation without advancing nuclear maturation. This effect is likely to be complex and mediated by direct effects on both the oocyte and cumulus cells. This knowledge will be of value to the creation of completely defined culture environments free of serum for oocyte maturation, which will improve both their overall safety and the efficacy of assisted reproductive technologies applied to both animals and humans.

CHAPTER 6

GENERAL CONCLUSION

6.1 GENERAL CONCLUSION

This thesis provides support for the hypothesis that the growth factors studied, namely activin, neurotrophin-4 and BDNF, play an essential role in the development and organisation of the human fetal ovary during the second trimester. There is also support, as evidenced by bovine *in vitro* maturation studies, that BDNF has a significant role in later oocyte development, enhancing cytoplasmic maturation and embryo development potential.

6.2 THE ROLE OF ACTIVIN IN HUMAN FETAL OVARIAN DEVELOPMENT

The studies described in Chapter 3 demonstrate the presence of activin subunits and activin/inhibin type I and type II receptors in the mid-trimester human ovary. Neither the inhibin subunit, nor its receptor, betaglycan, was identified by immunohistochemistry techniques. The activin binding protein, follistatin, was also not identified in the fetal ovary at this stage of development. These studies provide previously unpublished detail concerning presence of activin / inhibin in the developing human fetal ovary focussed around the time of follicular assembly.

Oogonia in the mid-trimester human fetal ovary were found to exclusively express the βA subunit. Furthermore, quantitative PCR studies determined a significant increase in βA expression over the mid-trimester. The effect of activin A on germ cell survival and proliferation was therefore investigated using a tissue culture model. *In vitro* supplement

with activin A resulted in a mean increase of 20% in the total number of germ cells, as well as a significant increase in germ cell BrdU immunostaining, indicating increased germ cell proliferation and/or entry into meiosis. Immunolocalisation of ActRIIA receptors specifically to germ cells, the global presence of ActRIIB and ALK4 across germ cell and non-germ cell compartments, and the stromal presence of ALK2, suggests that activin signalling can target germ cells both directly or indirectly, in an autocrine / paracrine manner.

These data demonstrate the presence and localisation of activin receptors and subunits within the developing human ovary, and support a role for activin A in the regulation of germ cell proliferation during the developmental period leading up to primordial follicle formation. This furthers our limited knowledge of the factors controlling germ cell development in the human fetal ovary, and ultimately contributes to our understanding of female fertility. Further work detecting and localising Smad downstream signalling molecules has been undertaken in adult human ovary (Pangas *et al.*, 2002), but not, as yet, in the human fetal ovary. Future studies clarifying Smad localisation, and possibly quantifying *in vitro* signalling responses, would generate further insight into the very complex subject of TGF- β superfamily signalling, and potentially identify areas amenable to therapeutic manipulation.

6.3 THE ROLE OF NEUROTROPHINS IN HUMAN FETAL OVARIAN DEVELOPMENT

The studies described in Chapter 4 have demonstrated the presence of the neurotrophins NT4 and BDNF, and their signalling receptors TrkB and p75, in the developing human ovary during the second trimester. This confirmed previous published data, with preferential location of ligands (BDNF to oocyte, NT4 to pre-granulosa) supporting autocrine / paracrine signalling. NT4 action was further studied by tissue culture experiments where the ligand was supplemented, blocked or substituted with BDNF, and the resulting effect on germ cell survival and proliferation examined.

Previous studies have shown decreased germ cell survival and proliferation when TrkB signalling is blocked using K252a, a potent and specific inhibitor of all Trk receptors (Spears *et al.*, 2003). In the same series of tissue culture experiments, inhibition of both NT4 and BDNF by use of blocking antibodies lowered murine oocyte survival, but blocking either ligand alone had no detectable effect (Spears *et al.*, 2003). In agreement, inhibition of NT4 in mid-trimester human fetal ovary culture experiments (using the same blocking antibody) showed no effect on germ cell survival. However, addition of BDNF in the presence of NT4 blocking antibody resulted in decreased germ cell survival, either by necrosis or apoptosis. It has long been recognised that massive germ cell loss occurs during human embryological development (Baker, 1963) and that a key feature in oocyte survival rests upon ovarian organisation and primordial follicle formation (Hirshfield, 1991; McLaren, 1991; Fulton *et al.*, 2005). It is possible that NT4, derived from granulosa cells, is an important growth factor for oocyte survival at

this time of primordial follicle assembly, and it is tempting to interpret these culture findings to suggest oocyte-derived BDNF may trigger oocyte apoptotic pathways, or render an oocyte vulnerable to necrosis, in the absence of NT4 signalling from surrounding granulosa cells.

This data supports the importance of a functional NT4 / BDNF / TrkB signalling complex in the developing human ovary, but leaves challenging questions concerning ovarian development as yet unanswered. It is intriguing that, whilst NT4 and BDNF presumably exist in some sort of balance, there is also a certain amount of plasticity in this growth factor system: there is little effect seen on germ cell number when one ligand is removed (blocked), supplemented, or removed and then replaced, possibly because alternative neurotrophins ligands can substitute in this scenario. Yet absence of both NT4 and BDNF, or their preferential receptor TrkB, results in significant oocyte loss (Spears *et al.*, 2003). Lack of NT4 in culture conditions supplemented with BDNF similarly result in germ cell necrosis or apoptosis, however, the converse situation (lack of BDNF replaced by NT4) would not appear to carry the same consequences (Fan *et al.*, 2000). The influence of stromal signalling via the p75 receptor, is also poorly understood. Clearly, further studies are needed to unearth the complexities of ovarian development.

A limiting factor in the acquisition of knowledge in this area of research is the relative scarcity of tissue and difficulty in obtaining samples. As a result, information is often gained by extrapolation from animal models. The unpredictable timing of sample

arrival, and potential delay in processing, is also a factor challenging efforts in this field. In time, as a greater bank of frozen and optimally fixed tissue samples are accumulated, it will be possible to continue and advance studies, thus gaining further knowledge concerning the role of these factors in human ovary development.

6.4 THE ROLE OF BDNF IN BOVINE OOCYTE MATURATION

Significant legal and ethical issues restrict studies of human oocyte maturation. The studies described in Chapter 5 therefore used a bovine model, selected on the basis of relative similarity to the human; cows are mono-ovulatory and polyestrous (rather than seasonal breeders). These studies have demonstrated the presence of the neurotrophin BDNF, and its signalling receptors TrkB and p75, in the bovine ovary and cumulus-oocyte complex. BDNF and p75 were also detected in immature and metaphase II arrested oocytes. Neither full length nor truncated TrkB could be detected in oocytes.

Bovine oocyte maturation was studied to determine a possible role for BDNF. Oocyte maturation entails nuclear maturation (progression to metaphase II, with extrusion of a single polar body) as well as cytoplasmic maturation (re-distribution and organisation of organelles in order to allow fertilisation, and molecular changes conferring developmental competence and the ability to become an embryo). Studies have shown that BDNF secretion is stimulated by the pre-ovulatory LH surge and suggest that it has a role in promoting oocyte maturation (Seifer *et al.*, 2002a; Kawamura *et al.*, 2005). BDNF supplementation of serum-free defined *in vitro* maturation media showed no

increase in MII (nuclear maturation) for either cumulus-enclosed oocytes or denuded oocytes. However, BDNF supplement during *in vitro* maturation resulted in an increased number of parthenogenetically activated oocytes forming blastocysts, implying enhanced cytoplasmic maturation. Denuded oocytes also showed increased cleavage rates and higher numbers of blastocysts when matured in the presence of BDNF (or serum), although these results were lower than maturation of intact COCs. Embryo cleavage was significantly lower in COC groups matured with BDNF blocking antibody, an effect that was not seen in identical maturation conditions using denuded oocytes. But no blastocysts were formed by denuded oocytes matured with BDNF blocking antibody, suggesting that BDNF may exert an influence directly on oocytes, as well as via cumulus.

These studies support a role for BDNF in oocyte cytoplasmic maturation, enhancing oocyte competence to form parthenogenetic blastocysts. Further studies fertilising, rather than activating, matured bovine oocytes would be useful to establish safety and optimal BDNF supplement concentrations. Studies using human oocytes, should also be undertaken, and, in time, it may be possible to create and refine defined serum-free *in vitro* maturation media for agricultural use as well as human infertility treatment.

In summary, this research has demonstrated that the various factors studied in the preceding chapters are unequivocally present in the mid-trimester human fetal ovary, and adult bovine ovary. These results alone imply essential roles in ovarian tissue proliferation and development. The discovery that activin A increases germ cell proliferation and/or entry into meiosis and the data supporting functional importance of NT4 / BDNF / TrkB signalling furthers our understanding of processes affecting the regulation of ovarian development at the time of follicular assembly. The discovery of the beneficial effects of BDNF on oocyte cytoplasmic maturation and resultant enhanced embryo developmental potential also contributes to our understanding of oocyte development. Further research developing ways of manipulating these factors *in vitro* will, hopefully, lead to therapeutic applications for this research.

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APPENDIX I

Materials

Sources of all reagents used for the experiments described in the preceding chapters are detailed below.

| HUMAN TISSUE CULTURE MATERIALS | SOURCE |
|---|---|
| 24-well culture plates | Costar Ltd, High Wycombe |
| Millicell CM filters | Millipore UK, Watford |
| Minimal essential medium (MEM α) | Gibco, Paisley |
| Bovine serum albumin (BSA), fraction V Electrophoresis grade | Sigma, Poole, Dorset |
| Penicillin | Sigma, Poole, Dorset |
| Streptomycin sulphate | Sigma, Poole, Dorset |
| Amphotericin | Sigma, Poole, Dorset |
| ITS | Sigma, Poole, Dorset |
| Glutamine | Sigma, Poole, Dorset |
| Pyruvate | Sigma, Poole, Dorset |
| Recombinant human Activin A | R & D Systems, Abingdon |
| Neurotrophin-4 (NT4) | Peprtech EC Ltd |
| Anti-neurotrophin-4 (anti-NT4) | Sigma, Poole, Dorset |
| Brain derived Neurotrophic Factor (BDNF) | Peprtech EC Ltd |
| Bromodeoxyuridine (BrdU) | Sigma, Poole, Dorset |
| Bouins fluid | Triangle Biomedical Sciences, Lancashire |

| BOVINE MATURATION CULTURE | SOURCE |
|---|-------------------------------------|
| PBS | Oxoid, Basingstoke, Hampshire |
| Universal container | Bibby Sterilin, Fisher Scientific |
| 10ml syringe | Becton Dickinson, Fisher Scientific |
| 18G needle | Becton Dickinson, Fisher Scientific |
| 90mm petri dish | Fisher Scientific |
| 4 well nunc plates | Nunc, Fisher Scientific |
| TCM199 | Sigma, Poole, Dorset |
| FSH | Ovagen |
| LH | Sigma, Poole, Dorset |
| Estradiol | Sigma, Poole, Dorset |
| Mycoplex fetal calf serum | PAA labs, Yeovil, Somerset |
| Brain derived Neurotrophic Factor (BDNF) | Peprtech EC Ltd |
| Anti- brain derived neurotrophic factor (Anti-BDNF) | Sigma, Poole, Dorset |
| HEPES buffered TCM (HB TCM) | See Appendix II for recipe |
| Bicarbonate buffered TCM (BB TCM) | See Appendix II for recipe |
| Mineral Oil | Sigma, Poole, Dorset |
| Hyaluronidase | Sigma, Poole, Dorset |
| BOVINE PARTHENOGENESIS | |
| Hepes buffered synthetic oviduct fluid (HSOF) | See Appendix II for recipe |
| Ionomycin | Sigma, Poole, Dorset |
| SOFaaBSA | See Appendix II for recipe |
| 6-DMAP | Sigma, Poole, Dorset |
| Cycloheximide | Sigma, Poole, Dorset |
| Hoescht 33342 | Sigma, Poole, Dorset |

| MOLECULAR BIOLOGY | SOURCE |
|--|--------------------------------------|
| RNAlater | Ambion, Huntingdon, Cambs |
| 0.2ml eppendorf tubes | Ambion, Huntingdon, Cambs |
| Deoxynucleotide triphosphate | Roche Diagnostics, Lewes |
| 2 x ThermoStart PCR mastermix | Abgene, Epsom |
| TBE buffer | See Appendix II for recipe |
| Agarose | Sigma, Poole, Dorset |
| Ethidium bromide | Sigma, Poole, Dorset |
| Gel tank | Biorad, CA, USA |
| Primer sequences | MWG Biotech |
| LightCycler Fast start DNA MasterSYBR Green 1 Master Mix | Roche Molecular Biochemicals, Sussex |
| ISOLATION OF RNA, SYNTHESIS AND AMPLIFICATION OF cDNA | |
| RNeasy mini kit | Qiagen, Crawley, Surrey |
| TRIReagent | Sigma, Poole, Dorset |
| chloroform | Sigma, Poole, Dorset |
| isopropanol | BDH (VWR) |
| DNAase | Gibco, Paisley |
| Deoxynucleotide triphosphate | Roche Diagnostics, Lewes |
| QIAshredder cell lysate homogeniser | Qiagen, Crawley, Surrey |
| Pellet paint | Novagen |
| Bulk first strand cDNA synthesis kit | Amersham Biosciences |
| dNTPs | Boehringer Mannheim, Lewes |

| IMMUNOHISTOCHEMISTRY | SOURCE |
|---|---|
| Slides | BDH Laboratory supplies |
| Histoclear | National Diagnostics, Atlanta, Georgia, USA |
| Hydrogen Peroxide (H ₂ O ₂) | Merck Ltd (BDH), Lutterworth, UK |
| Methanol | Merck Ltd (BDH), Lutterworth, UK |
| Tris base | Sigma, Poole, Dorset |
| Tris-buffered saline (TBS) | See Appendix II for recipe |
| Blocking serum | Diagnostics Scotland, Carlisle, UK |
| Citrate buffer | See Appendix II for recipe |
| Bovine serum albumin, fraction V, electrophoresis grade | Sigma, Poole, Dorset |
| Avidin block | Vector, Peterborough, UK |
| Biotin block | Vector, Peterborough, UK |
| Biotinylated secondary antibodies | Dako, Cambridge, UK |
| Biotinylated anti-chicken secondary antibody | Jackson Laboratories, Bar Harbour, Maine, USA |
| Avidin-Biotin complex-horseradish peroxidase | Dako, Cambridge, UK |
| DAB liquid substrate-chromagen system | Dako, Cambridge, UK |
| Haematoxylin | See Appendix II for recipe |
| Eosin | See Appendix II for recipe |
| Type I rat-tail collagen | Sigma, Poole, Dorset |
| 0.1M acetic acid | BDH laboratory supplies |
| TCM199 | Sigma, Poole, Dorset |
| IMMUNOCYTOCHEMISTRY | |
| Microtubule stabilising buffer | See Appendix II for recipe |
| Triton x100 | BDH Laboratory supplies |
| 37% formaldehyde | BDH Laboratory supplies |
| 10% goat serum | See Appendix II for recipe |
| 5% goat serum | See Appendix II for recipe |
| 0.1% goat serum | See Appendix II for recipe |
| Rhodamine phalloidin | Sigma, Poole, Dorset |
| Alpha anti-tubulin FITC | Sigma, Poole, Dorset |
| Vectashield and DAPI | Vector, Peterborough |

| SPECIFIC IMMUNOHISTOCHEMISTRY | SOURCE |
|---|--|
| Inhibin α subunit (179.3k) | |
| Mouse monoclonal antibody | Gift of NP Groome |
| Inhibin / activin β A subunit (E4) | |
| Mouse monoclonal antibody | Gift of NP Groome |
| Inhibin / activin β B subunit (13/14) | |
| Mouse monoclonal antibody | Gift of NP Groome |
| Inhibin / activin β B subunit (β B (80-112)-NH2 PBL#197) | |
| Rabbit polyclonal antibody | Gift of W Vale |
| ActRIIA | |
| Rabbit polyclonal antibody | Gift of C-H Heldin |
| ActRIIB | |
| Rabbit polyclonal antibody | Gift of C-H Heldin |
| ALK2 | |
| Rabbit polyclonal antibody | Gift of C-H Heldin |
| ALK4 | |
| Rabbit polyclonal antibody | Gift of C-H Heldin |
| Betaglycan | |
| Goat polyclonal antibody | Santa Cruz, CA, USA |
| Follistatin (FS288) | |
| Mouse monoclonal antibody | Gift of NP Groome |
| BrdU | |
| Mouse monoclonal antibody | Roche Diagnostics, Lewes |
| BDNF (N20) | |
| Rabbit polyclonal antibody | Santa Cruz, CA, USA |
| BDNF peptide block | Santa Cruz, CA, USA |
| NT4 | |
| Rabbit polyclonal antibody | Santa Cruz, CA, USA |
| TrkB | |
| Chicken polyclonal antibody | Promega, Southampton |
| Truncated TrkB | |
| Rabbit polyclonal antibody | Santa Cruz, CA, USA |
| p75 | |
| Mouse monoclonal antibody | Strattech Scientific Ltd, Soham, Cambs |

APPENDIX II

Recipes for solutions used in previously described experiments are detailed below. All chemicals were from Sigma, Poole, UK unless otherwise stated.

MOLECULAR BIOLOGY SOLUTIONS

10X TBE

108 g tris base

55 g boric acid

3.77 g EDTA

Add milliQ water to a final volume of 1.0L

GEL FOR PCR ELECTROPHORESIS

MINIGEL

50 ml TBE

0.5 g agarose

Mix in conical flask and heat for 2 minutes in microwave until agarose dissolved

Pour into gel cast with comb in situ and allow to set

150 ml GEL

150ml TBE

1.5 g agarose

Method as above

IMMUNOHISTOCHEMISTRY SOLUTIONS

CITRATE BUFFER (0.1M, pH 6.0)

42.02 g citric acid (monhydrate)

1900 ml distilled water

Add concentrated NaOH to pH 6.0

Make up to 2.0L with further distilled water

Use as 0.01M, diluting in distilled water

TRIS BUFFERED SALINE (TBS) (0.5M, pH 7.5)

121.1 g tris (trizma base)

800 ml distilled water

Add concentrated HCl to pH 7.5

Make up to 2.0L with further distilled water

IMMUNOCYTOCHEMISTRY SOLUTIONS**PHOSPHATE BUFFERED SALINE (PBS)**

1 tablet per 100 ml milliQ water (Oxoid, Basingstoke, Hampshire)

5XSB

7.55 g Pipes (0.1M)

0.25 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (5mM)

0.235 g EGTA (2.5mM)

Add 10N NaOH to pH 6.9

Final volume 50 ml

Store at 4°C for 1 year

0.1% NGS WASH SOLUTION

50 ml goat serum

50 ml PBS

Store at 4°C for 1 month

5% NGS WASH SOLUTION

2.5 ml goat serum

47.5 ml PBS

0.01 g Na Azide

50 ml Triton X100

Store at 4°C for 1 month

10% NGS WASH SOLUTION

5 ml goat serum

72.5 ml PBS

0.015 g Na Azide

75 ml Triton X100 (BDH)

Store at 4°C for 1 month

MICROTUBULE STABILISING BUFFER (MTSB)

1 ml 5XSB

5 ml DTT (1M)

2.5 ml D₂O (Deuterium oxide)

1.5 ml MilliQ water

COMPLEX FIX

5 ml MTSB

5 ml Triton X100

Warm to 37°C 30 minutes before use

Warm 270 ml formaldehyde (37% stock) and add just before use

ANTIBODY SOLUTION

400 ml 5% NGS

5 ml rhodamine phalloidin

8 ml alpha α -tubulin FITC

Incubate in dark at 37°C for 1 hour

BOVINE CULTURE MEDIA STOCKS

STOCK B (250 mM Sodium Bicarbonate)

1.05 g NaHCO₃

Add MilliQ water to 50 ml

Add 2 - 3 grains of phenol red

Osmolarity 430-440 mOsM

Filter and store at 4°C for up to 3 weeks

STOCK C (33 mM Sodium Pyruvate)

0.036 g Pyruvic Acid

Add MilliQ water to 10 ml

Osmolarity 55-60 mOsM

Filter and store at 4°C for up to 1 week

STOCK D (171 mM Calcium chloride)

1.26 g CaCl₂·2H₂O

Make to 50 ml with MilliQ dH₂O

Osmolarity 430-440 mOsM

Store filtered at 4°C for 3 months

STOCK G (60 mM Glucose)

0.54 g Glucose

Make to 50 ml with MilliQ dH₂O

Osmolarity 60-70 mOsM

Store filtered at 4°C for 3 months

STOCK GLN (10 mM Glutamine)

0.0438 g L-Glutamine

Make to 30 ml with MilliQ dH₂O

Osmolarity 8 mOsM

Store filtered at 4°C for 1 week

STOCK H (250 mM Hepes)

3.0 g Hepes Free Acid

3.25 g Hepes Sodium Salt

Add MQ water to 100 ml

Add 2 - 3 grains of phenol red

Osmolarity 380-385 mOsM

Filter and store at 4°C for up to 1 month

STOCK L (330 mM Sodium Lactate)

11.75 ml Na Lactate (60%) Syrup

Make to 250 ml with MilliQ dH₂O.

Osmolarity 590-640 mOsM

Filter and store at 4°C for up to 3 months

STOCK M

0.5 g MgCl₂.6H₂O

Make to 50 ml with MilliQ dH₂O

Osmolarity 120-130 mOsM

Store filtered at 4°C for 3 months

STOCK S2

3.145 g NaCl

0.267 g KCl

0.081 g KH₂PO₄

0.0125 g Kanamycin

Make to 50 ml with MilliQ dH₂O

Osmolarity 2173 mOsM

Store filtered at 4°C for 3 months

2X TCM 199 STOCK

20 ml TCM 199 (x10)

0.01 g Kanamycin

0.04 g L-Glutamine

Add MilliQ water to 100 ml

Filter and store at 4°C for up to 1 week

BOVINE MATURATION MEDIA STOCKS

HEPES BUFFERED TCM199 (100 ml)

50 ml 2X TCM 199 Stock

6 ml Stock H

2 ml Stock B

42 ml MilliQ water

Check osmolarity (280-300 mOsM)

Check pH (7.3-7.4)

Filter and aliquot into 9 ml, store at -20°C

HB TCM199 9ml

Add 1ml fetal calf serum

Filter sterilise

Use for COC / oocyte sorting and washes

BICARBONATE BUFFERED TCM 199 (50 ml)

25 ml 2X TCM 199 Stock

5 ml Stock B

0.3 ml Stock C

19.7 ml MilliQ water

Osmolarity 280-300 mOsM

Check pH (7.3-7.4)

Filter and aliquot into 5 ml, store at -20°C

IN VITRO MATURATION BASE MEDIUM (serum free)

5 ml BB TCM199

50 ml FSH (final concentration 0.5 mg/ml)

20 ml LH (final concentration 5 mg/ml)

Filter sterilise

Add 2 µl estradiol (final concentration 2µg/ml)

Use for COC / oocyte wash and wells in IVM culture plates

BOVINE PARTHENOGENIC ACTIVATION MEDIA

HEPES SOF (+ CALCIUM) (HSOF)

| | |
|--------------|---------|
| STOCK S2 | 2.0 ml |
| STOCK B | 0.4 ml |
| STOCK H | 1.6 ml |
| STOCK C | 0.2 ml |
| STOCK D | 0.2 ml |
| STOCK M | 0.2 ml |
| STOCK L | 0.2 ml |
| STOCK G | 0.5 ml |
| MilliQ water | 14.7 ml |

Adjust pH to 7.40. Osmolarity 265-275 mOsM

Filter and store 1 - 2 weeks at 4°C

SOFaaBSA CULTURE MEDIUM

| | |
|---------------------------|----------|
| STOCK S2 | 1.5 ml |
| STOCK B | 1.5 ml |
| STOCK C | 0.15 ml |
| STOCK D | 0.15 ml |
| STOCK M | 0.15 ml |
| STOCK L | 0.15 ml |
| STOCK G | 0.375 ml |
| STOCK GLN | 1.5 ml |
| Non Essential amino acids | 0.15 ml |
| Essential amino acids | 0.3 ml |
| MilliQ water | 9.075 ml |
| Fatty acid free BSA | 0.12 g |

Adjust pH to 7.40. Osmolarity 265-275 mOsM

Filter and store 1 week at 4°C

HYALURONIDASE

Add 1 ml MilliQ water to 30 mg vial

Divide into 10 µl aliquots (=300U) and store at -20°C

Final concentration used is 300U/ml

Add 1 ml HSOF to 10 µl aliquot

IONOMYCIN

Add 1338.5 µl DMSO to 1mg to make 1mM stock

Add 25.46 µl MilliQ water to make 0.05mM working stock

Divide into 100 µl aliquots and store at -20°C

Final concentration used is 5mM

Add 50µl to 450 µl HSOF

6-DIMETHYLAMINOPURINE (6-DMAP)

20mg 6-DMAP

Add 4.9 µl MilliQ water to make 25mM working stock

Divide into 100 µl aliquots and store at -20°C

Final concentration used is 2.5mM

Add 50µl to 450 µl SOFaaBSA

CYCLOHEXIMIDE

Add DMSO to make 100mg/ml stock solution

Add 1.99ml DMSO to 10µl stock solution to make 500µg/ml working stock

Divide into 20 µl aliquots and store at -20°C

Final concentration used is 10µg/ml

Used in combination with 6-DMAP

Add 10µl to 500µl medium (50µl DMAP + 450µl SOFaaBSA)

APPENDIX III

PUBLICATIONS

Expression of activin subunits and receptors in the developing human ovary: activin A promotes germ cell survival and proliferation before primordial follicle formation

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Abstract

The formation of the essential functional unit of the ovary, the primordial follicle, occurs during fetal life in humans. Factors regulating oogonial proliferation and interaction with somatic cells before primordial follicle formation are largely unknown. We have investigated the expression, localisation and functional effects of activin and its receptors in the human fetal ovary at 14–21 weeks gestation. Expression of mRNA for the activin β A and β B subunits and the activin receptors ActRIIA and ActRIIB was demonstrated by RT-PCR. Expression of β A mRNA increased 2-fold across the gestational range examined. Activin subunits and receptors were localised by immunohistochemistry. The β A subunit was expressed by oogonia, and the β B subunit and activin receptors were expressed by both oogonia and somatic cells. β A expression was increased in larger oogonia at later gestations, but was low in oocytes within newly formed primordial follicles. Treatment of ovary fragments with activin A *in vitro* increased both the number of oogonia present and oogonial proliferation, as detected by bromodeoxyuridine (BrdU) incorporation. These data indicate that activin may be involved in the autocrine and paracrine regulation of germ cell proliferation in the human ovary during the crucial period of development leading up to primordial follicle formation.

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Keywords: Activin; Fetal ovary; Oocyte; Primordial follicle; Growth factor

Introduction

Germ cell survival is key to the reproductive life span of the ovary and is determined by the continuing presence of oocytes within primordial follicles. These are formed during fetal life in the human. The number of follicles formed is dependent upon the balance between early germ cell proliferation and loss, as well as the interaction between oocyte and somatic cells (Hirshfield, 1991; McLaren, 1991). The pathways regulating this crucial process are largely unknown, although several genes necessary for the formation of a normal complement of primordial follicles have been identified (Amleh and Dean, 2002; Matzuk, 2000). During the period leading up to primordial follicle formation there is

massive oogonial mitotic proliferation, both during and after migration from the yolk sac to the nephrogonadoblastic ridge. The oogonia associate with somatic cells resulting in syncytial nests of germ cells intermingled with pre-granulosa cells and surrounded by a meshwork of ovarian stroma (Byskov, 1986; Pepling and Spradling, 2001; Sawyer et al., 2002). The germ cells subsequently enter meiosis only to arrest at diplotene of the first meiotic division. By mid-gestation, the human fetal ovary contains its maximal number of germ cells. This peak is followed by widespread germ cell loss, by apoptosis, (De Pol et al., 1997; Vaskivuo et al., 2001) so that less than 20% survive by the time of birth (Baker, 1963). Primordial follicles are formed as oocytes become individually surrounded by somatic cells (Hirshfield, 1991) and it is this interaction that has been identified as the event that determines oocyte survival, and presumably protection against apoptosis. A number of locally derived growth factors have been identified to be crucial to germ cell survival at early developmental stages, such as BMP-4 (Fujiwara et al., 2001) and kit ligand (Godin et al., 1991;

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anova et al., 1990), and following primordial follicle formation, such as growth and differentiation factor 9 (GDF-9) (Dong et al., 1996). However, little information is available regarding factors regulating oogonial proliferation and primordial follicle formation, particularly in human.

Activins and inhibins are members of the transforming growth factor β (TGF β) family. The component α and β subunits can be combined into biologically active $\alpha\beta$ inhibin and $\beta\beta$ activin dimers with generally opposing functional effects, and are produced in both gonad and pituitary (Burger and Igarashi, 1988; Chen, 1993; Mather et al., 1997). Follistatin is a structurally unrelated monomeric protein that reversibly binds activin and neutralises its biological effects (Takamura et al., 1990; Schneyer et al., 1994). Members of the TGF superfamily can influence many facets of cell lineage and activity including proliferation, differentiation, adhesion, motility and apoptosis. Many are involved in embryonic development as well as adult tissue homeostasis (Mishina et al., 1999; Padgett and Patterson, 2001). The activins, together with other members of the TGF β superfamily, signal through membrane-bound serine-threonine kinase receptors. They bind to a type II receptor (ActRIIA or ActRIIB), which recruits and phosphorylates a type I receptor with subsequent modulation of gene expression via Smad protein activation (Massagué, 1998). Several type I receptors, termed activin receptor-like kinases (ALKs) have been identified. ALK4 (ActRIB) is believed to be the referential activin receptor, whereas ALK2 (ActRIA) preferentially mediates BMP and anti-Müllerian hormone signalling. Whilst the Smad proteins are the central elements in the activin receptor signalling pathway, other signalling pathways may mediate at least some of the diverse biological responses of the TGF β superfamily, including mitogen-activated protein kinase (MAPK) pathways (Massagué, 2000; Mulder, 2000). The initial steps coupling the activin type II serine-threonine kinase receptor to MAPK are yet to be defined, although signalling via TGF β -activated kinase 1 (TAK1) results in activation of stress-activated p38 and transcription factor ATF2, which can interact with Smad3 and Smad4 to mediate transcription (Cocolakis et al., 2001; Derynck et al., 2001).

A substantial body of evidence has accrued that activins and inhibins are regulatory factors in the adult ovary involved in a wide range of functions, including folliculogenesis, granulosa and theca cell proliferation, and steroidogenesis (Findlay et al., 2002; Knight and Glister, 2001; Mizunuma et al., 1999) as well as oocyte maturation (Alak et al., 1998; Izadyar et al., 1998). Activin is expressed in the human gonad at very early stages of development (Harkness and Baird, 1997) and there are limited data indicating the expression of activin and inhibin in the developing human ovary following primordial follicle formation (Harkness and Baird, 1997; Rabinovici et al., 1991). However, no data are available regarding the presence of activin and its cognate receptors before primordial follicle formation in the human ovary. We have therefore explored

the expression and distribution of inhibin/activin subunits in the human fetal ovary during the period of oogonial proliferation leading up to primordial follicle formation. The localisation of activin receptors ALK4, ActRIIA and ActRIIB, the inhibin receptor betaglycan (Lewis et al., 2000) and the activin-binding protein follistatin has also been investigated to identify potential sites of action of these regulatory factors during this period of development. As oogonia in the mid-trimester human fetal ovary were found to express activin subunits and receptors, we have investigated the effect of activin A on oogonial survival and proliferation using an *in vitro* tissue culture model.

Materials and methods

Tissues

Human fetal ovaries were obtained following medical termination of pregnancy. Women gave consent according to national guidelines (Polkinghorne, 1989) and the study was approved by the Lothian Paediatrics/Reproductive Medicine Research Ethics Subcommittee. Termination of pregnancy was induced by treatment with mifepristone (200 mg orally) followed 48 h later by prostaglandin E1 analogue (Gemeprost; Beacon Pharmaceuticals, Tunbridge Wells, UK) 1 mg three hourly per vaginum. None of the terminations were for reasons of fetal abnormality, and all fetuses appeared morphologically normal. Gestational age was determined by ultrasound examination before termination and confirmed by subsequent direct measurement of foot length.

Ovaries were dissected free and either fixed for immunohistochemical analysis, snap frozen and stored at -70°C , placed in 500 μl RNeasy Lysate (Qiagen (Europe) Ltd, Huntingdon, Cambs, UK) or further dissected and placed into culture. Fixation was carried out in Bouin's fluid for 5 h, followed by transfer to 70% ethanol before processing into paraffin using standard methods.

Isolation of RNA and synthesis and amplification of cDNA

Total RNA was extracted from fetal ovary (14–19 weeks) using either the RNeasy Mini Kit (Qiagen, Crawley, UK) for RT-PCR or TRIzol Reagent (Sigma, Poole, Dorset, UK) for quantitative PCR analysis according to the manufacturers' instructions, and treated with DNase (Gibco, Paisley, UK). Reverse transcription using a first-strand cDNA synthesis kit (Roche Diagnostics, Lewes, UK) was followed by PCR using 1 μl cDNA samples and Taq DNA polymerase (AGS Gold; Hybaid, Ashford, UK) as previously described (Robinson et al., 2001). Primers were designed to span an intron to ensure that genomic DNA was not amplified. Specific primers for each subunit and receptor are given in Table 1. Control tubes were run in parallel for each PCR, one in which water replaced RNA and a second RT-sample in which reverse transcriptase was omitted, to ensure there was no

Table 1
Sequences of PCR primers

| Primers | 5' | 3' |
|---------------------------|-------------------------|-----------------------|
| ActRIIA | GCAAAATGAATACGAAGTCTA | GCACCCTCTAATACCTCTGGA |
| ActRIIB | CAACTTCTGCAACGAGCGCTT | GCGCCCCGAGCCTTGATCTC |
| Inhibin α | TGAGGGCCCTGTTCTTGGATG | CTGGCGGTGCGTGTATGCTG |
| Inhibin/activin β A | GAACCTATGGAGCAGACCTCGG | TTGCCATCACACTCCAAGCC |
| Inhibin/activin β B | GCCAGGAGCGCGTTTCCGAAATC | CCGCTCGCCCCGCTCAAACAA |
| GAPD | GACATCAAGAAGGTGGTGAAGC | GTCCACACCCTGTTGCTGTAG |

genomic DNA contamination. The identity of all PCR products were confirmed by direct sequencing using an Applied Biosystems 373A automated sequencer.

Lightcycler quantitative PCR

Quantitative PCR was performed using the Lightcycler system (Roche Molecular Biochemicals, Sussex, UK) as previously described (Hartley et al., 2002) to investigate changes in expression of the activin β A subunit over a range of gestations. Reverse transcribed RNA samples ($n = 29$) were diluted in water as indicated. One microliter of diluted first-strand cDNA was added to a final volume of 10 μ l containing 2 mM $MgCl_2$ and 0.5 μ M each of forward and reverse primer in $1 \times$ Lightcycler Fast Start DNA MasterSYBR Green 1 Master Mix (Roche Molecular Biochemicals). Signal acquisition was performed for each of 45 amplification cycles followed by continuous melt curve analysis to ensure product accuracy. Primers for GAPD and activin β A subunit are given in Table 1.

Standard curves for GAPD and activin β A were derived by making a series of first-strand cDNA dilutions from a 16-week ovary. The number of cycles needed to yield a fluorescent signal above background (the cross-over point, C_p) was plotted against the log of relative concentration using LightCycler Software (Molecular Dynamics Ltd., Buckinghamshire, UK) yielding a straight line for each product. Quantification of ovarian β A mRNA expression was subsequently performed. For each experiment, both GAPD and activin β A amplification reactions were performed in duplicate for every cDNA sample used. Calculations for activin β A mRNA concentration were made relative to GAPD from the same sample to allow comparisons between ovaries. Allowance for differences in amplification rate for GAPD and activin β A was achieved by determining the actual amount of amplification required to yield a signal for each target. Data were analysed by ANOVA.

Immunohistochemistry

Sections (5 μ m) were mounted on BDH Superfrost Plus slides (BDH Laboratory Supplies, Merck Ltd, Lutterworth, Leics, UK) and dried overnight at 50°C before processing for immunohistochemistry as previously described (Anderson et al., 2002). Briefly, slides were incubated in 3%

H_2O_2 in methanol for 30 min to inhibit endogenous peroxidase activity. After rinsing in distilled water, slide were washed twice in Tris-buffered saline (TBS; 0.05 M Tris, 0.85% NaCl, pH 7.6) for 5 min and blocked for 3 min in appropriate serum (Diagnostics Scotland, Carlisle, UK) diluted 1:5 in TBS containing 5% bovine serum albumin. Sections were then blocked with avidin and biotin (both from Vector, Peterborough, UK) with washes in TBS in between. Various primary antibodies were used. Rabbit antibodies to Activin receptor II (ActRIIA) and Activin receptor IIB (ActRIIB) were kindly donated by Dr. C-H Heldin, Ludwig Institute for Cancer Research, Uppsala, Sweden, and used at a concentration of 1:60 and 1:400, respectively. Rabbit antibodies to ALK2 and ALK4 were also kindly donated by Dr. C-H Heldin, Ludwig Institute for Cancer Research, Uppsala, Sweden and both used at concentration 1:150. Goat antibody to betaglycan (Santa Cruz Biotechnology, CA, USA) was used at a dilution of 1:25. Mouse monoclonal primary antibodies to the inhibin/activin subunits included α subunit (173.9k) used at a range of dilutions 1:1000 to 1:100, β A subunit (E4) at 1:1000 and β B subunit (12/13) at 1:100, all the gift of NP Groome. Monoclonal antibody directed against recombinant follistatin FS288, which recognises follistatin even when bound to activin, was used at a range of dilutions 1:200 to 1:25, also gift of NP Groome (Majdic et al., 1997). All were incubated at 4°C overnight. Sections were then washed and incubated for 30 min with goat anti-rabbit (Santa Cruz Biotechnology), rabbit anti-goat (Dako, Cambridge, UK) or rabbit anti-mouse (Diagnostics Scotland) biotinylated secondary antibody as appropriate, at a dilution of 1:500. Following washes in TBS sections were incubated with avidin-biotin-horseradish peroxidase linked complex (Dako) according to the manufacturer's instructions. Bound antibody was visualised using 3,3'-diaminobenzidine tetrahydrochloride (Dako). Nonimmune serum was used in place of primary antibody for negative controls. Positive controls (human fetal testis for activin/inhibin subunits and receptors and adult rat testis for follistatin) were also run in parallel.

Sections were counterstained with haematoxylin, dehydrated, mounted and visualised by light microscopy. Images were captured using an Olympus Provis microscope (Olympus Optical Co., London) equipped with Kodak DCS330 camera (Eastman Kodak Co, Rochester, NY).

Nuclear diameter measurement

The size distribution of germ cells expressing activin β A subunit was determined in specimens of 18–19 weeks gestation. To avoid bias, sequential nonoverlapping frames were captured using the Provis image analysis system, and the diameter of all germ cell nuclei within the frame measured. Strongly immunostained germ cells were classified as β A positive, all other germ cells as negative. Data were analysed by the Mann–Whitney *U* test as the distribution deviated from normal. For graphical representation, numbers of immunopositive and immunonegative cells were grouped in 1- μ m increments of nuclear diameter. The nuclear diameter of a total of 527 germ cells was measured.

Primary cell culture

Fetal ovaries were dissected and cultured in MEM α medium with phenol red (GibcoBRL, Life Technologies) supplemented with 2 mM pyruvate, 2 mM glutamine, 1 \times ITS supplement (Sigma) and 3 mg/ml BSA, and with penicillin, streptomycin and amphotericin. A total of seven ovaries were used for culture experiments. Fetal ovaries (14–17 weeks) were dissected in prewarmed and gassed medium to yield tissue fragments approximately 1 mm³. Between four and six fragments were transferred in 400- μ l medium to each of four designated wells containing inserts (Millicell-CM; Millipore UK Ltd, Watford, UK) in a 24-well tissue culture plate such that tissue fragments were supported at the meniscus of the medium. Tissue was cultured for 17 h in a humidified incubator at 37°C and 5% CO₂. Two treatment groups were cultured with 100 ng/ml recombinant human activin A (R&D systems, Abingdon, UK). The other two groups acted as controls. Bromodeoxyuridine (BrdU; Sigma) was supplemented at 10 μ M/ml as a marker of cell proliferation. In each experiment, BrdU was present in the media of one control and one treatment group for the duration of the experiment. BrdU was added to the other control and treatment group for the final 4 h of tissue culture only. Analysis of germ cell number in these parallel cultures allowed confirmation that changing the medium 4 h before the end of the culture period did not influence the results. At the end of culture, tissues were washed in PBS and fixed in Bouin's fluid for 24 h, followed by processing to visualise BrdU incorporation by immunohistochemistry.

Serial sections (5 μ m), four sections per slide, were mounted on BDH Superfrost Plus slides (BDH Laboratory Supplies) and dried overnight at 50°C. Every fourth slide was processed for immunohistochemistry as described above. The primary antibody used was formalin-grade anti-BrdU mouse monoclonal antibody (Roche) at a dilution of 1:30, incubated at 4°C overnight. On completion of the immunohistochemistry protocol, sections were counterstained with haematoxylin, dehydrated and mounted before analysis.

Sections of uncultured control and cultured tissue were analysed to quantify the effects of culture and activin A on the number and proliferation of germ cells. Analysis was carried out blind using the Area Fraction Probe in the Stereologer software programme (Systems Planning and Analysis Inc, Alexandria, VA, USA) as previously described (Robinson et al., 2003; Sharpe et al., 2002). Tissue was serially sectioned and sections greater than 20 μ m apart were counted, ensuring the same cells were not counted more than once. The counting was performed using a 121 point grid in the eyepiece of the microscope. Each tissue fragment was outlined and frames randomly selected thereafter by the programme. The number of points lying outside the tissue in any grid was also recorded and the total cell numbers were corrected for this. Average number of germ cells per grid was calculated for each experimental condition, and data were analysed using paired *t* tests.

Results

Expression of mRNAs for activin/inhibin subunits and receptors

mRNA expression was detected by RT-PCR using RNA extracted from human fetal ovaries (Fig. 1). Amplicons of expected sizes corresponding to the inhibin α (278 bp), inhibin/activin β A (274 bp) and β B (325 bp) subunits and the activin receptors ActRIIA (435 bp) and ActRIIB (283 bp) were amplified from cDNA derived from ovaries across 14–19 weeks gestation. Product identity was confirmed by direct sequencing.

Quantitative PCR

Changes in the expression of the activin β A subunit mRNA between 14 and 19 weeks gestation were investigated by quantitative RT-PCR. There was an approximately 2-fold increase in the relative expression of the β A subunit mRNA expression between 14 and 19 weeks gestation ($P < 0.001$, Fig. 2). The increase in expression was not continuous across the gestational range examined, but mostly occurred between 17 and 19 weeks.

Immunohistochemistry

Inhibin/Activin subunits

Expression of the β A inhibin/activin subunit protein was detected in the fetal ovary at all gestations investigated. It was specifically localised to the germ cell cytoplasm at all gestations studied, but a marked change in the pattern of immunostaining was seen across the gestational range (Figs. 3D–F). At 14 weeks gestation, a gradient of distribution was observed. Oogonial immunostaining was detected throughout the ovary, but was most marked at the periphery, particularly in germ cells in the outermost few layers of the

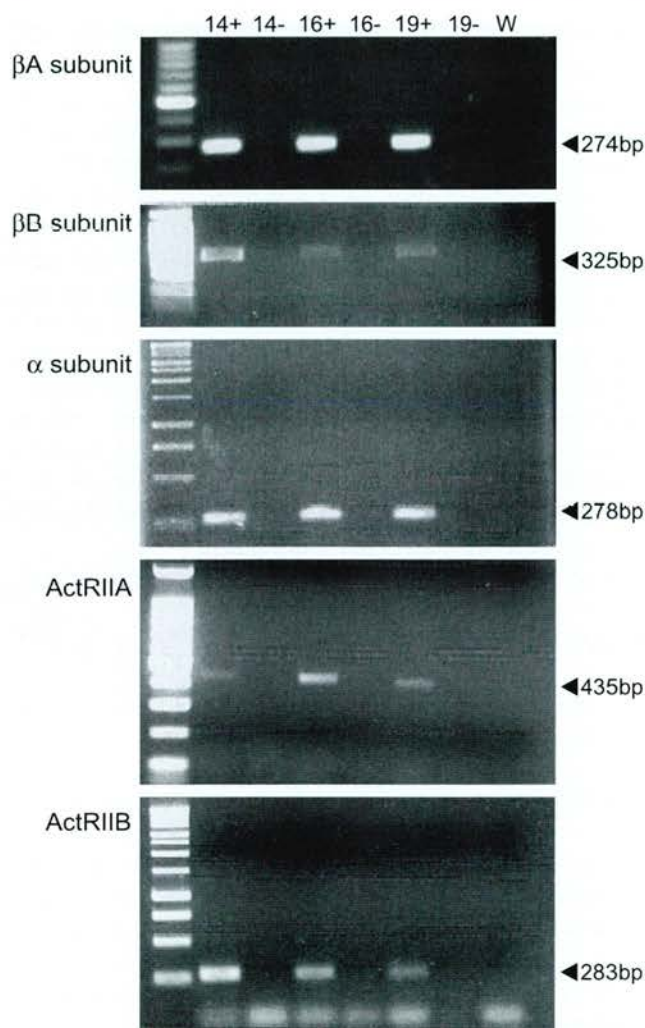


Fig. 1. Expression of mRNA for inhibin/activin subunits and type II activin receptors in the human fetal ovary. RT-PCR analysis of samples extracted from human fetal ovaries at 14, 16 and 19 weeks gestation. mRNA expression for inhibin/activin subunits and receptors ActRIIA and ActRIIB as indicated. Lanes marked 14–, 16– and 19– contained samples in which reverse transcriptase was omitted. W refers to water instead of cDNA in the PCR reaction. The size of each amplicon is indicated on the right.

gonad. At 17–19 weeks gestation, a different distribution of immunostaining had emerged with a discrete subpopulation of germ cells showing intense staining. These germ cells tended to be larger oocytes, and were distributed in small clusters of less than 10 largely located in the medullary region of the ovary. However, oocytes within newly formed primordial follicles showed only weak or no immunostaining for activin β A.

Differential expression of β A subunit by larger germ cells was confirmed by direct measurement of nuclear diameter. A total of 527 germ cells were measured, of which 79 (15%) were intensely immunopositive for the β A subunit. These β A-expressing germ cells were significantly larger than those only weakly immunopositive or immunonegative (11.3 ± 0.2 vs. $9.8 \pm 0.1 \mu\text{m}$, $P = 0.0001$, Fig. 4) although the very largest germ cells with nuclear

diameter $>16 \mu\text{m}$, that is, those within primordial follicle were immunonegative.

Expression of β B inhibin/activin subunit protein was also detected throughout the ovary across the range of gestations examined. Immunostaining was widespread, with both germ cells and stromal cells expressing the β B subunit (Figs. 3G–I). However, at the periphery of the ovary, some germ and somatic cells did not express the β B subunit. There was no difference in distribution of immunostaining of either stromal or germ cells between 14 and 18 week gestation. Two antibodies directed against the β B subunit (C12/13 monoclonal antibody, gift of NP Groome, and a cyclic inhibin β B (80-112)-NH₂ PBL#197 rabbit polyclonal, gift of W Vale) were used, with similar results.

Immunostaining for the inhibin α subunit was not detected in any cell type in the human fetal ovary across a range of gestations and despite using a wide range of antibody titres. Fetal testes were used as a positive control and showed immunostaining in both Leydig cells in the interstitium and Sertoli cells in the seminiferous tubules as previously described (Figs. 3A–C) (Anderson et al. 2002).

Activin and inhibin receptors

Expression of the type II receptor ActRIIA was detected at all gestations from 14 to 21 weeks and was localised specifically to germ cell cytoplasm (Figs. 5C, D). There was no immunostaining in stromal cells or the pre-granulosa

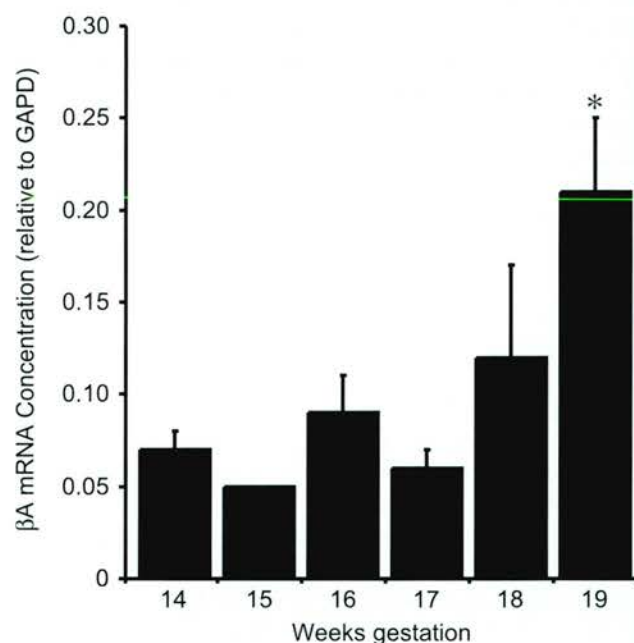


Fig. 2. Real-time PCR quantification of β A expression in the fetal ovary. Expression of β A subunit mRNA was quantified in human fetal ovary specimens over the gestational range 14–19 weeks. Data calculated as β A mRNA expression relative to GAPDH for ovaries of 14, 15, 16, 17, 18 and 19 weeks gestation ($n = 6, 5, 5, 5, 3$ and 5 , respectively, mean \pm SEM). SEM for 15 weeks = 0.00. * $P < 0.001$ by ANOVA.

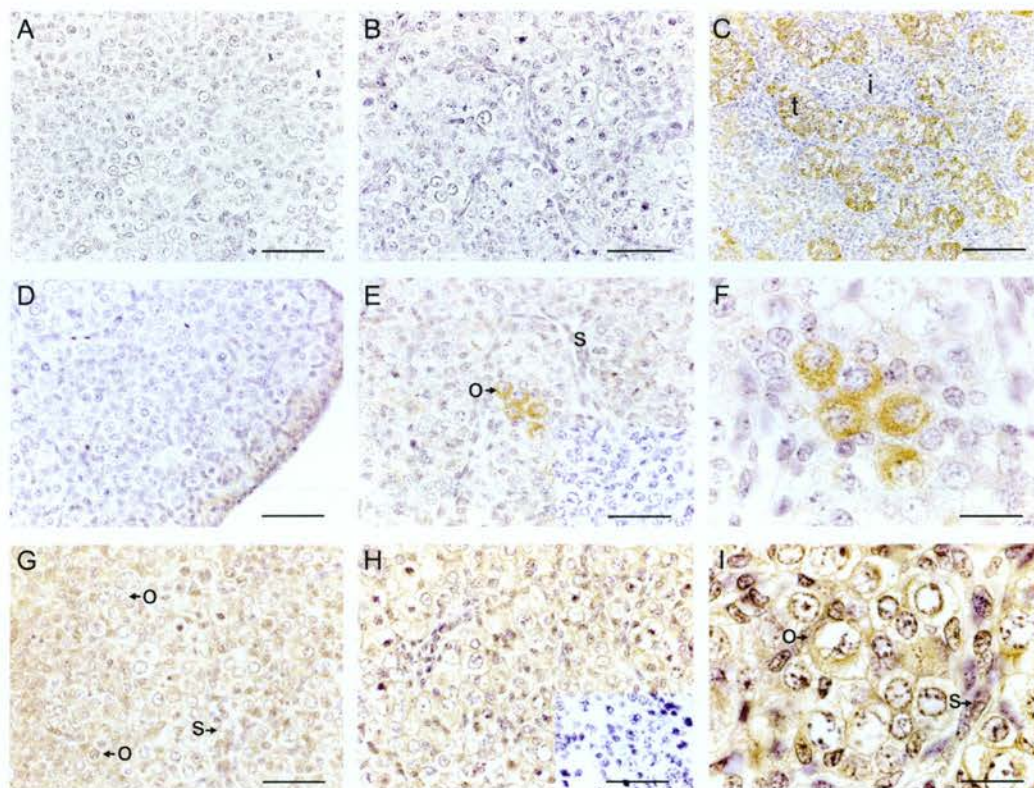


Fig. 3. Immunohistochemical localisation of inhibin/activin subunits in human fetal ovary. (A) Inhibin α subunit, 14-week ovary. (B) Inhibin α subunit, 19-week ovary. (C) Inhibin α subunit positive control, 19-week testis. (D) βA subunit, 14-week ovary. (E) βA subunit, 17-week ovary (inset shows negative control). (F) βA subunit, 17-week ovary at higher magnification. (G) βB subunit, 14-week ovary. (H) βB subunit, 18-week ovary (inset shows negative control). (I) βB subunit, 18-week ovary at higher magnification. Positive staining is brown and sections are counterstained with haematoxylin. o, oogonia; s, stroma; t, tubule; i, interstitium. Scale bars in A–H, 50 μ m, F and I, 20 μ m.

cells of primordial follicles, nor cells at the ovarian surface. There was no significant difference in distribution across the gestational range studied.

ActRIIB immunorexpression was distributed widely within the fetal ovary at all gestations from 14 to 21 weeks (Figs. 5E, F). Immunostaining was detected in both stromal and germ cells but not in the cells at the ovarian surface. Intense immunostaining was noted in larger germ cells at later gestations and expression persisted in cytoplasm of oocytes organising into primordial follicles.

Expression of ALK4 protein, the preferential activin type I receptor, was detected throughout the ovary predominantly in stromal and pre-granulosa cells but also with weak expression in germ cells (Fig. 5A). At 14 weeks gestation, ALK4 immunostaining clearly demarcated a meshwork of ovarian stromal cells with weaker immunostaining of the oogonia intermingled with more strongly immunopositive pre-granulosa cells. At later gestations (18 weeks), the ovarian stroma was less strongly immunopositive, and the predominant site of ALK4 expression was the pre-granulosa cells surrounding individual and grouped oogonia, although oogonia remained weakly immunopositive.

In contrast to ALK4, expression of ALK2 protein was exclusively localised to the stromal cells at 14–18 weeks

gestation, with no immunostaining identified in germ cells at any gestation (Fig. 5B). Intense immunostaining was particularly notable in many pre-granulosa cells between and

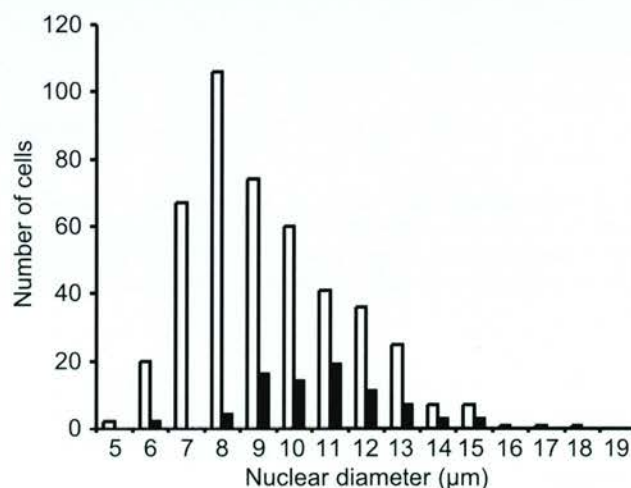


Fig. 4. Size distribution of βA -immunopositive germ cells in the human fetal ovary. Histogram shows the frequency of distribution of nuclear diameter of cells strongly immunopositive for βA subunit (filled columns) and those either weakly immunopositive or immunonegative for βA subunit (open columns) in human fetal ovary at 18–19 weeks gestation. $n = 79$ and $n = 448$, respectively.

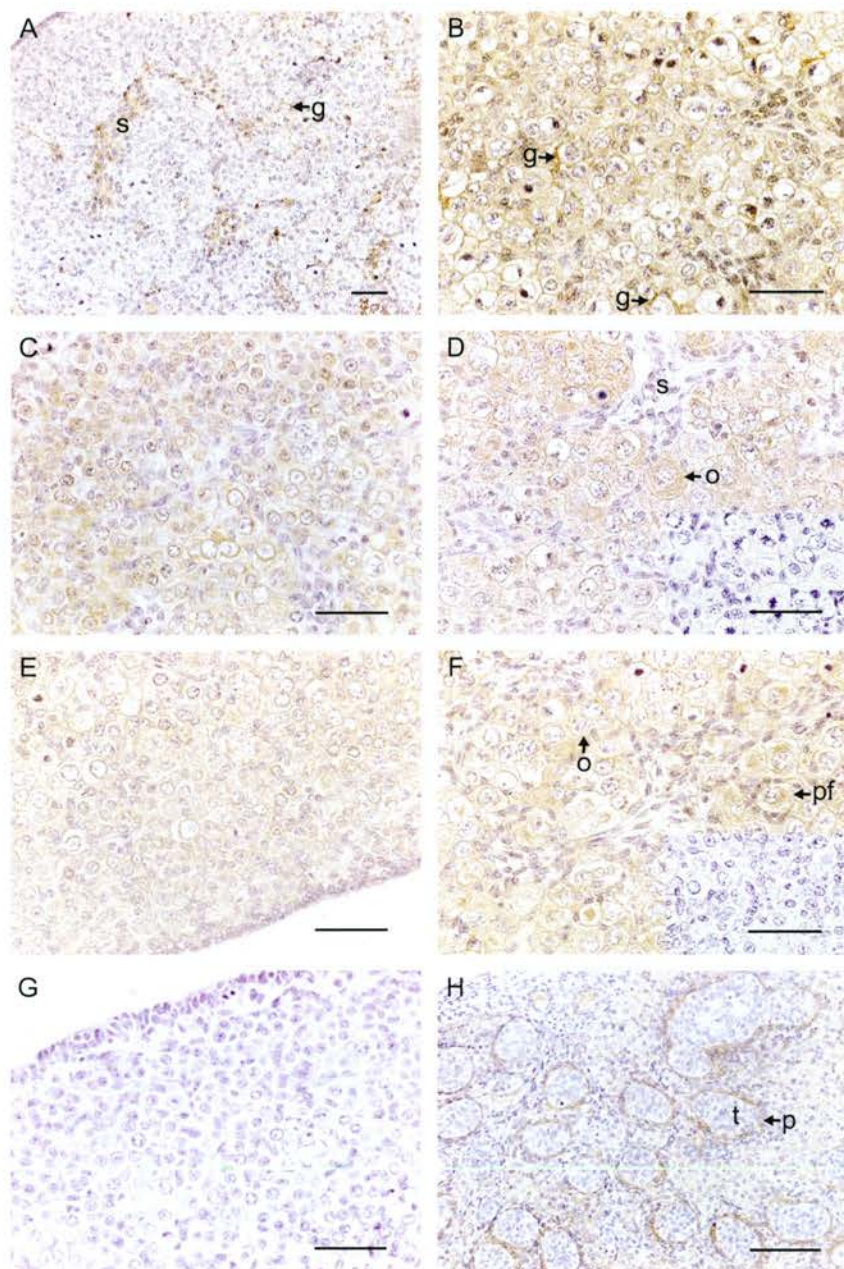


Fig. 5. Immunohistochemical localisation of inhibin/activin receptors in human fetal ovary. (A) ALK2, 14-week ovary. (B) ALK4, 18-week ovary. (C) ActRIIA, 14-week ovary. (D) ActRIIA, 21-week ovary (inset shows negative control). (E) ActRIIB, 14-week ovary. (F) ActRIIB, 21-week ovary (inset shows negative control). (G) Betaglycan, 14-week ovary. (H) Betaglycan-positive control, 15-week testis. Positive staining in all panels is brown and sections are counterstained with haematoxylin. s, stroma; g, pre-granulosa cells; o, oogonia; pf, primordial follicle; t, tubule; p, peritubular cells. Scale bars, 50 μ m in all panels.

outlining germ cells. This pattern of stromal cells immunostaining appeared to increase with increasing gestation and the formation of primordial follicles.

No expression of β -glycan protein was detected in any mid-trimester gestation ovary studied. Intense immunostaining was detected in the peritubular cells of fetal testes used as a positive control (Figs. 5G, H).

In all cases, negative controls showed no immunostaining. Fetal testis sections were used as a positive control,

and showed distinct immunostaining as previously described (Anderson et al., 2002).

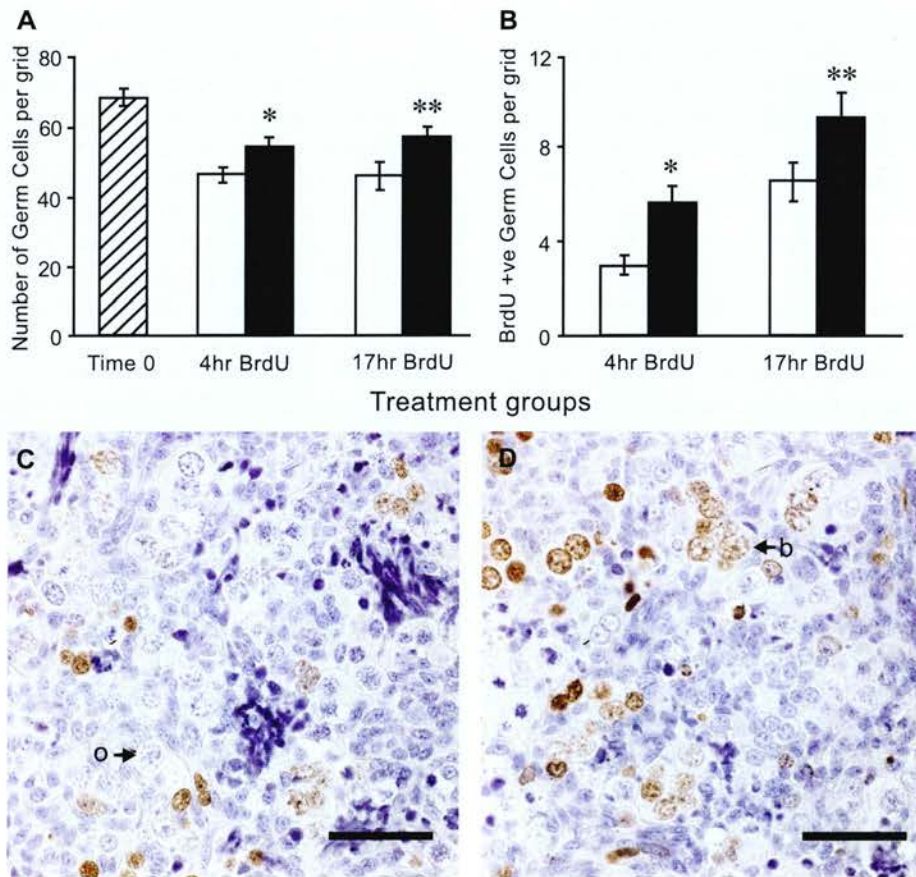
Follistatin

No expression of follistatin was detected in any mid-trimester ovary studied (14–20 weeks gestation). Adult rat testis was used as a positive control and showed positive immunostaining in Leydig cells as previously described (Majdic et al., 1997).

(17 h), more germ cells were immunopositive than when BrdU was present for only the final 4 h of culture, as expected (6.4 ± 1 vs. 2.8 ± 0.4 stained germ cells per grid when exposed to BrdU for only 4 h). Activin A treatment also resulted in a significant increase in number of BrdU-immunopositive germ cells. There was a 44% increase in number of immunostained germ cells (9.2 ± 1.3 vs. 6.4 ± 0.8 per grid, $P = 0.01$) when cultured with activin A and BrdU for 17 h. Immunostained germ cells were increased by 89% (5.3 ± 0.9 vs. 2.8 ± 0.4 per grid, $P < 0.05$) when cultured with activin A for 17 h and exposed to BrdU for only the final 4 h of culture (Figs. 6B, C).

Discussion

High interspecies conservation of activins, inhibins and activin receptors and the universal presence of activins in mammals, birds, amphibians and fish suggest an evolution-



arily conserved role of these proteins in animal development. Activins have potent effects on early amphibian embryonic events during mesoderm-forming (Nieuwkoop) induction both *in vitro* and *in vivo* (Dyson and Gurdon, 1997). In mammals, activins have also been demonstrated to have morphogenetic effects, notably in renal tubule development (Ritvos et al., 1995). However, there are few published data available concerning the presence, expression and potential roles of activins, inhibins and their receptors during mammalian gonadal development. Whilst activin is thought to act primarily in an autocrine and/or paracrine manner, inhibin acts primarily as an endocrine feedback regulator of pituitary FSH release (Welt et al., 2002). Our data demonstrate the presence and localisation of activin receptors and subunits within the developing human ovary, and suggest a role for activin A in the regulation of germ cell proliferation during the developmental period leading up to primordial follicle formation. In contrast, follistatin was not expressed. Follistatin binds and neutralises activin activity. It also binds several other TGF β superfamily members, including BMP4 (Fainsod et al., 1997; Lin et al., 2003). Follistatin expression has been detected in the fetal sheep ovary at late gestation, but not before primordial follicle formation (Braw-Tal et al., 1994). Similarly, follistatin is not expressed in the developing human or rat testis (Majdic et al., 1997) despite the presence of activin subunits and receptors (Anderson et al., 2002). It would therefore appear that activin activity in the developing human gonad is not regulated by follistatin.

Transgenic mice have been generated to study physiological models deficient in inhibins, activins and their receptors (Burns and Matzuk, 2002). Gene knockout experiments have been inconclusive in determining activin function *in vivo*, but functional analysis would suggest that activins are not essential for mammalian mesoderm formation. A number of reasons are cited to explain the lack of definitive conclusions from this body of work; firstly, some mutations are lethal, secondly, deficient activin ligand or receptor signalling results in a phenotype with suppressed FSH which results in reproductive and developmental defects and thirdly, there may be some functional overlap between TGF β superfamily ligand and receptor signalling. Mutations in the gene encoding the β A subunit are lethal. Mutant mice develop to term but have secondary palate defects and lack whiskers and lower incisors (Matzuk et al., 1995b). The mutant mice die within 24 h of birth due to feeding difficulties and there are thus no conclusions regarding ovarian follicular development. Mice lacking the β B subunit suffer eye lesions as a result of failed eyelid fusion. Mutant females do not show a disruption in follicular development but manifest impaired reproduction characterised by perinatal lethality of their offspring (Vassalli et al., 1994). Highly increased β A expression was noted in the ovaries of these β B-deficient female mice, suggesting the possibility of functional compensation within the TGF β superfamily. Using a gene knockin approach, it has been

found that activin β B can rescue the craniofacial defects and neonatal lethal phenotype of β A-deficient mice. However, activin β B is unable to substitute for all the functions of β A. The β B knockin mouse is subfertile and phenotypic abnormalities in the gonads and external genitalia, as well as delayed lethality remain in these mice. Ovaries in the hemizygous knockin female mouse are much smaller than those of controls (Brown et al., 2000). This may suggest activin β A expression is necessary for development of the correct complement of germ cells in the mouse ovary, consistent with the present data in the human. It is interesting that mice carrying a null mutation in the *ActRII* gene do not, as one might expect, phenocopy activin-deficient mice. Despite lacking a crucial component of the activin signalling pathway, only 25% of these mice die at or shortly after birth with mandible defects. Surviving adults have defective reproductive performance and small gonads although this may be in part due to abnormal FSH secretion in addition to deficient germ cell proliferation (Matzuk et al., 1995a). There is also some evidence that it is the second messenger (Smad) signalling which is more crucial to specific embryonic and extra-embryonic development pathways than particular ligand and receptor interactions (Chang et al., 2001).

Activin subunits and receptors showed a differential distribution within the developing ovary. Activins bind to a type II receptor (*ActRIIA* or *ActRIIB*), which recruits and phosphorylates a type I receptor with subsequent modulation of gene expression via Smad protein activation (Masagué, 1998). Several type I receptors (ALKs) have been identified. ALK4 (*ActRIB*) is believed to be the preferential activin receptor. The activin β A subunit was specifically expressed by germ cells, whereas both somatic and germ cells expressed the β B subunit. *ActRIIA* was specifically localised to germ cells, whereas *ActRIIB* and the type I receptor ALK4 were expressed by both stromal and germ cells. The distinct distribution of both activin subunits and receptor subtypes between germ cells and somatic cells suggest that activin may have several distinct roles in ovarian development and organisation, and more specifically in germ cell regulation. This is supported by observations of an increase in β A mRNA expression as well as changes in the pattern of β A protein expression across the mid-trimester. With increasing gestation, intense β A expression was found in small clusters of larger, more mature germ cells. However, the largest oocytes, within primordial follicles, show little or no β A expression. This pattern suggests that the increase in β A subunit expression may be transient as the germ cells mature, with reduction of gene expression following primordial follicle formation. Increased expression however may be associated with differences in subsequent survival, consistent with the effects of activin on oogonial survival as demonstrated in the tissue culture experiments here described.

Despite detecting inhibin α subunit mRNA by RT-PCR, we were unable to demonstrate the protein by immunohistochemistry. Previous studies suggest that activin but not

inhibin is present in the human fetal ovary at mid-gestation (16–23 weeks) (Rabinovici et al., 1991). Rabinovici et al. localised the β A subunit to primordial follicles by immunohistochemistry, but did not observe immunostaining for β B or α subunits. In contrast, immunostaining for all three subunits was detected in late gestation fetal rhesus monkey ovary, which contained primordial and early growing follicles. Furthermore, culture medium from mid-gestation human fetal ovaries was found to contain undetectable levels of inhibin- α by radioimmunoassay. Recent immunolocalisation studies performed in baboons revealed minimal to nondetectable levels of α subunit in mid- and late-gestation ovaries (Billiar et al., 2003) and there is also indirect evidence from *in situ* hybridisation studies on primate ovaries that follicles preferentially synthesise activin rather than inhibin in the early stages of development (Schwall et al., 1990; Yamoto et al., 1993). The putative inhibin receptor betaglycan (Lewis et al., 2000) was also not detected in the mid-trimester human fetal ovary. The fact that neither the α subunit nor betaglycan receptor were identified by immunohistochemistry in mid-trimester human fetal ovaries indicates that inhibin is not a regulatory factor at this stage of development, but becomes of importance later during follicular growth (Welt et al., 2002).

An *in vitro* tissue culture model was used to investigate the effect of activin on oögonial survival and proliferation. Activin A treatment resulted in an increase in germ cell proliferation and survival in fetal ovary tissue fragments. This concurs with the earlier demonstration that activin A stimulated [3 H] thymidine incorporation *in vitro* in the differentiating rat ovary (Kaipia et al., 1994). BrdU was used as a marker for cell proliferation. It is interesting to note that immunostaining was observed predominantly towards the edge of cultured ovarian fragments. This may represent a diffusion effect, but may also partly reflect the organisation of the tissue. Mitotically dividing germ cells tend to be placed peripherally in the mid-trimester fetal ovary with a gradient of maturity across the developing ovary such that relatively more mature cells are found in the medulla compared to the cortex (Byskov, 1986). A recent study of follicle formation during fetal development in sheep also noted a cortical pattern of distribution of BrdU staining following *in vivo* administration (Sawyer et al., 2002), which became more pronounced up to primordial follicle formation.

In the adult ovary, activin A induces expression of genes signalling cellular differentiation and maturation. In the developing fetal ovary, key events involve germ cell proliferation, organisation and survival by somatic cell interaction and primordial follicle formation (Hirshfield, 1991; McLaren, 1991). The present data support a role of activin A in germ cell survival and proliferation. The Bcl-2 family of proteins are key regulators of apoptosis in many cell types. This family comprises pro-apoptotic (Bax, Bad and Bak) and anti-apoptotic (Bcl-2, Bcl-X_L and Mcl-1) subfamilies. Mcl-1 is a rapidly inducible short-term effector of cell

viability and is expressed in a subset of oocytes at the time of primordial follicle formation (Hartley et al., 2002). The distribution of Mcl-1 in the fetal ovary is similar to that of activin β A subunit and it is possible that activin A promotes cell survival by signalling via Mcl-1, as has been demonstrated in a leukaemia cell line (Fukuchi et al., 2001). Whilst both activin A and Mcl-1 expression are most marked in larger oogonia preceding primordial follicle expression, oocytes within primordial follicles continue to express Mcl-1, whereas activin β A expression is low once that developmental stage is reached. It is possible that activin and Mcl-1 constitute part of the signalling pathway that determines survival for a small proportion of the oogonia in the mid-trimester ovary.

In conclusion, we have found the human fetal ovary to be a site of expression of activin subunits and receptors before the formation of primordial follicles. Protein expression of the inhibin α subunit and the betaglycan receptor was not detected. The distribution of activin subunits and receptors suggests that whilst somatic cells may produce any of the activin forms, oogonia only produce activin A, and is consistent with an autocrine and/or paracrine mode of action for activins within the developing ovary. Increased expression of β A by larger/more mature oogonia may reflect selection for follicle formation and data from tissue culture experiments support activin A as a regulatory factor in fetal germ cell proliferation and survival. These data suggest that activin may be of particular importance during the period of development immediately preceding primordial follicle formation.

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Brain-derived neurotrophic factor promotes bovine oocyte cytoplasmic competence for embryo development

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Abstract

The ability of an oocyte to support early embryonic development requires both nuclear and cytoplasmic maturation. We have investigated the effects of brain-derived neurotrophic factor (BDNF) on maturation of the bovine oocyte and embryo development after parthenogenetic activation. By RT-PCR and immunohistochemistry, cumulus and oocytes were shown to express mRNA and protein for BDNF and the p75 common neurotrophin receptor. However, mRNA for the BDNF-specific full length and truncated isoforms of the TrkB receptor are only detected in cumulus, suggesting that oocytes and cumulus differ in their capacity to respond to neurotrophin signalling. In *in vitro* maturation experiments, the proportion of cumulus oocyte complexes maturing to metaphase II was not altered by BDNF in groups lacking fetal calf serum (FCS), but was significantly lower than the positive control containing 10% FCS ($P < 0.01$). However, after maturation, the proportion of parthenogenetically activated oocytes forming blastocysts was highest for 10 ng/ml BDNF (24%, $n = 95$) followed by 100 ng/ml BDNF (18%, $n = 91$) and 10% FCS (15%, $n = 103$), which in turn were greater than no serum (10%, $n = 83$; $P < 0.01$). Maturation in the presence of a BDNF blocking antibody resulted in a blastocyst yield that was comparable to the absence of serum, and lower than in the presence of BDNF ($P < 0.01$). Similar effects on progression to metaphase II and blastocyst formation were observed using oocytes matured without cumulus. Together, these results provide the first evidence for a role for neurotrophins in promoting oocyte cytoplasmic competence to support embryonic development, despite being insufficient in the absence of serum to enhance nuclear maturation.

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Introduction

Oocyte competence to support development after fertilisation or parthenogenetic activation is acquired gradually over the course of oogenesis and folliculogenesis and is completed during meiotic maturation (Eppig 2001, Liu & Aoki 2002, Liu *et al.* 2003). At this time the meiotic segregation of chromatin is accompanied by critical changes in oocyte cytoplasm that remain poorly defined. The significance of these changes is illustrated by the wide differences observed in embryo developmental competence following variations in oocyte *in vitro* maturation culture conditions (Keskintepe & Brackett 1996, Krisher & Bavister 1999, Watson *et al.* 2000). A broad range of factors has been found to improve meiotic maturation *in vitro* and subsequent embryo developmental potential. These include supplementation of culture media with follicular fluid or serum, or specific gonadotrophins, steroid and thyroid hormones, retinoids, and different energy substrates and nutrients. These can benefit oocytes directly or

via cumulus cells (see reviews by Sutton *et al.* (2003), Chian *et al.* (2004)). Specific growth factors identified as intra-ovarian regulators of oocyte maturation that have been shown to be beneficial to bovine oocyte developmental competence in *in vitro* studies include epidermal growth factor (EGF), insulin-like growth factor I (IGF-I), activin A, inhibin A, and midkine, a heparin-binding growth factor (Lonergan *et al.* 1996, Stock *et al.* 1997, Rieger *et al.* 1998, Silva & Knight 1998, Ikeda *et al.* 2000).

There is increasing evidence of a role for neurotrophins in ovarian development and function, including oocyte maturation. Neurotrophins are a family of related growth factors initially identified to be important for regulation of neuronal survival and differentiation, but which have also been described in a variety of non-neuronal tissues including the cardiovascular, immune, endocrine and reproductive systems (Matsuda *et al.* 1988, Polak *et al.* 1993). They include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophins 3 and 4 (NT3

and NT4). Neurotrophins are unique in that they utilise two different receptors to mediate their biological actions: tyrosine kinase (Trk) receptors encoded by the *trk* proto-oncogene family (Trk A–C and truncated isoforms), and the p75 receptor, a member of the tumour necrosis factor (TNF) receptor superfamily (Bibel & Barde 2000, Rabizadeh & Bredesen 2003). The p75 receptor is widely expressed and binds all neurotrophins. By contrast, the Trk receptors show selective affinity for different neurotrophins (i.e. TrkA for NGF, TrkB for BDNF and NT4 and TrkC for NT3). Splice variants and truncated isoforms of Trk receptors lacking intracellular tyrosine kinase domains have also been identified (Bibel & Barde 2000).

BDNF and TrkB have been identified in the adult avian ovary (Jensen & Johnson 2001) and NT4 expression has also been localised to the oocyte in both rodent (Dissen *et al.* 1995) and *Xenopus* (Ibanez *et al.* 1992). TrkB expression appears to be central to the normal formation of primordial follicles that occur in the ovary in the few days following birth in the rodent (Spears *et al.* 2003), and for oocyte survival during early follicular growth (Paredes *et al.* 2004), an effect that may be predominantly mediated by truncated TrkB receptors. Mice carrying a null mutation of the NGF gene show deficient development of primordial follicles (Dissen *et al.* 2001). A direct effect of BDNF on murine oocyte maturation *in vitro* has also been reported, with increased first polar body extrusion rate in oocytes stripped of cumulus prior to maturation (Seifer *et al.* 2002a). BDNF is present in human follicular fluid (Seifer *et al.* 2003), and there is evidence for increased secretion of BDNF by cumulus cells, but not mural granulosa cells, in response to cAMP (Seifer *et al.* 2002a). These data suggest that cumulus-derived BDNF may be involved in oocyte maturation, and it is possible that its production is stimulated by gonadotrophins.

In the present study we have investigated the effects of BDNF on maturation of the bovine oocyte as well as implications for embryo development after parthenogenetic activation. Parthenogenesis provides a means of assessing oocyte cytoplasmic competence to elicit development independently of sperm mediated factors, and is an accepted standard to assess oocyte viability for cloning and nuclear reprogramming (De Sousa *et al.* 2002, Liu & Aoki 2002). We provide evidence that cumulus cells and oocytes may have different capacities to respond to neurotrophin signalling, and that neurotrophin signalling during maturation benefits oocyte cytoplasmic competence but not nuclear maturation.

Materials and Methods

Tissues

Bovine ovaries were collected from a local abattoir and kept warm during transportation. In the laboratory, ovaries were washed in Dulbecco's phosphate-buffered saline (PBS; Oxoid Ltd, Basingstoke, Hampshire, UK) at 38 °C, after which follicles measuring 4–8 mm were aspirated.

For immunohistochemistry, all tissue was fixed in Bouin's fluid overnight, and transferred to 70% ethanol before paraffin embedding and sectioning. To facilitate their handling prior to fixation, cumulus oocyte complexes (COCs) were first embedded in 50 µl droplets of collagen solution prepared by dissolving 4.2 mg type I rat-tail collagen (Sigma, Poole, UK) in 1 ml 0.1 M acetic acid (BDH) and mixing with an equal volume of 2 × TCM199 (Sigma), pH 7.2, immediately before use. Droplets containing COCs were then incubated at 37 °C for 10 min to set (Izadyar *et al.* 1998). For RNA isolation, ovaries were diced to 5 mm³ cubes and COCs, denuded oocytes and cumulus were snap frozen and stored at –70 °C.

Extraction of RNA and synthesis and amplification of cDNA

Total RNA was extracted using a RNeasy mini kit (Qiagen, Crawley, UK) as previously described (Young *et al.* 1998). Reverse transcription using a bulk first strand cDNA synthesis kit (Amersham Biosciences, Bucks, UK) was followed by PCR on 2 µl cDNA samples using 2 × thermostart PCR mastermix as per manufacturers instructions (Abgene, Epsom, UK). Specific primers for each gene are given in Table 1. For each gene, negative controls to confirm the absence of genomic DNA consisted of PCR on RNA without performing first-strand cDNA synthesis (RT–), and water. A further control tube was included and run in parallel as a blank for Qiagen reagents (non-embryo control). The identity of all PCR products was confirmed by direct sequencing using an Applied Biosystems 373A automated sequencer (Applied Biosystems, Foster City, CA, USA).

Immunohistochemistry

Immunohistochemistry was performed on sections of whole ovary and COCs. Sections (5 µm) were mounted on BDH Superfrost Plus slides (BDH Laboratory Supplies, Merck Ltd, Lutterworth, UK) and dried overnight at 50 °C before processing for immunohistochemistry as previously described (Anderson *et al.* 2002). Briefly, slides were incubated in 3% H₂O₂ in methanol for 30 min to inhibit

Table 1 PCR primer sequences.

| Gene ^a | Primer | Sequence | Product size (bp) |
|-------------------|---------|-------------------------------|-------------------|
| BDNF | Forward | 5'-CATGGGACTCTGGAGAGCAT-3' | 198 |
| | Reverse | 5'-CAAAGGCACTTGACTGCTGA-3' | |
| Trk B | Forward | 5'GGCCAGATGCTGTCTATTAT-3' | 206 |
| | Reverse | 5'-TCCTGCTCAGGACAGAGGT-3' | |
| Truncated Trk B | Forward | 5'CATTGTTACCAATCACACGGAGTA-3' | 430 |
| | Reverse | 5'CCATCCAGTGGGATCTTATGAAA-3' | |
| p75 | Forward | 5'TGGACAGCGTGACCTTCTC-3' | 153 |
| | Reverse | 5'-TCGTCTCGTCTGGTAATAGC-3' | |

^a Gene specific primers were designed based on homology to Genbank sequences specified by the following accession numbers: BDNF X97914; Trk B, NM_006180; Truncated Trk B, AF508964; p75, (AF228020).

endogenous peroxidase activity. After rinsing in distilled water, slides were washed twice in Tris-buffered saline (TBS; 0.05 M Tris, 0.85% NaCl, pH 7.6) for 5 min and blocked for 30 min in serum (Diagnostics Scotland, Carlisle, UK) diluted 1:5 in TBS containing 5% bovine serum albumin. Sections were then blocked with avidin and biotin (both from Vector, Peterborough, UK) with washes in TBS in between. The following primary antibodies were used: BDNF (N20) (rabbit polyclonal; Santa-Cruz Biotechnology, CA, USA) which was used at 1:100 as previously described (Schneider *et al.* 2001); TrkB (chicken polyclonal; Promega, Southampton, UK) used at 1:25; p75 (mouse monoclonal; Neomarkers, Soham, Cambridgeshire, UK) used at 1:25. All were incubated at 4°C overnight. Sections were then washed and incubated for 30 min with swine anti-rabbit (Dako, Cambridge, UK), donkey anti-chicken (The Jackson Laboratory, Bar Harbor, ME, USA) or rabbit anti-mouse (Dako) biotinylated secondary antibody as appropriate, at a dilution of 1:500. Following washes in TBS, sections were incubated with avidin-biotin-horseradish peroxidase linked complex (Dako) according to the manufacturer's instructions. Bound antibody was visualised using 3,3'-diaminobenzidine tetrahydrochloride (Dako). Non-immune serum was used in place of primary antibody for negative controls as well as BDNF blocking peptide (sc-546 P, Santa Cruz Biotechnology), whilst rat brain was used as a positive control tissue. Sections were counterstained with haematoxylin, dehydrated, mounted and visualised by light microscopy. Images were captured using an Olympus Provis microscope (Olympus Optical Co., London, UK) equipped with Kodak, DCS330 camera (Eastman Kodak Rochester, NY, USA).

Oocyte maturation in vitro

Follicular aspirate was maintained at 38°C and allowed to settle. The cellular debris was then transferred to a petri-dish containing HEPES-buffered TCM199 (Sigma) with 10% (heat inactivated) FCS to allow sorting and selection of COCs. All procedures were carried out in a laminar flow hood using a Leica microscope (Leica, Wetzlar, Germany) with heated stage at 38°C. COCs were transferred through 3 × 1.5 ml volumes of HEPES-buffered TCM199 with 10% FCS before transfer in a minimal volume to 1.5 ml of base maturation medium for random allocation into maturation treatment groups. The base medium for cumulus enclosed and denuded (see below) oocyte maturation consisted of bicarbonate-buffered TCM199 supplemented with 0.01 IU/ml follicle stimulating hormone (FSH) (Ovagen: ICPbio, Auckland, New Zealand), 0.125 IU/ml LH (Sigma) and 2 µg/ml oestradiol (Sigma). *In vitro* maturation treatments were comprised of culturing 15–25 oocytes in 500 µl volume of base medium with no additional supplements (i.e. serum-free negative control), 10% FCS (positive control), 10–100 ng/ml of recombinant human BDNF (PeproTech EC Ltd, London, UK), 5 µg/ml monoclonal

anti-human BDNF (Sigma) or both 10 ng/ml recombinant human BDNF and 5 µg/ml monoclonal anti-human BDNF.

COCs were cultured at 38.5°C in a humidified incubator with 5% CO₂. After 26 h maturation culture COCs were stripped and either processed for immunocytochemistry to assess meiotic progression or activated to produce parthenogenetic embryos. Cumulus was stripped from oocytes by a combination of mechanical vortex and incubation in 300 IU/ml hyaluronidase (Sigma) for 60–90 seconds in serum-free HEPES buffered Synthetic Oviduct Fluid (HEPES SOF) consisting of 108 mM NaCl, 7.2 mM KCl, 1.2 mM KH₂PO₄, 5 mM NaHCO₃, 20 mM HEPES, 0.33 mM Na-Pyruvate, 1.7 mM CaCl₂, 0.5 mM MgCl₂, 3.3 mM Na-Lactate, 1.5 mM glucose, 3 mg/ml BSA (fatty acid free), pH 7.4, osmolarity 265–275 (Thompson *et al.* 1995). Enzymatic action was neutralised by transferring oocytes through subsequent washes of HEPES SOF⁺ supplemented with 10% FCS.

In a second group of experiments oocytes were stripped of cumulus prior to maturation, and only completely denuded oocytes were selected, and washed in serum-free base maturation medium (see above). Five cumulus-free maturation treatments were evaluated consisting of base medium with no additional supplements (i.e. serum-free), 10% FCS, 10 ng/ml of recombinant human BDNF, 5 µg/ml monoclonal anti-human BDNF or both 10 ng/ml recombinant human BDNF and 5 µg/ml anti-human BDNF. In each experimental replicate, a sixth group of cumulus enclosed oocytes were matured in base maturation media supplemented with 10% FCS (positive control). As previously, oocytes were cultured at 38.5°C in a humidified incubator with 5% CO₂ for 26 h and subsequently either processed for immunocytochemistry to assess meiotic progression or activated to produce parthenogenetic embryos.

Immunocytochemical analysis of meiotic progression

Maturation to metaphase II (MII) was assessed by immunocytochemical staining of microtubules to visualise spindle morphology, of microfilaments to visualise cortical membranes and segregation of the first polar body, and DAPI to visualise condensed chromatin. Stripped oocytes were fixed and immunostained for microtubules and microfilaments using a modification of the method described by Messinger & Albertini (1991). Microtubule stabilising buffer comprising 5 × SB (0.1 M Pipes (Sigma), 5 mM MgCl₂, 2.5 mM EGTA pH 6.9 in NaOH), 1 M DTT, deuterium oxide (Aldrich, Gillingham, UK) and distilled water was incubated with Triton X100 (BDH) and 37% formaldehyde to make complex fix. Stripped oocytes were incubated in complex fix for 30 min at 37°C and then washed 3 times in 0.1% goat serum before blocking in 10% goat serum for 1 h at room temperature. Oocyte groups were then incubated in the dark at 37°C for 1 h in 50 µl droplets of 5% goat serum with rhodamine phalloidin and anti-tubulin FITC, washed 3 times in 10% goat serum and then mounted in Vectashield and DAPI (Vector). Oocytes were partially squashed with a

coverslip that was then sealed using clear nail varnish. Slides were visualised using fluorescent microscopy and each oocyte scored for meiotic progression. Oocytes were scored as MII arrested if they exhibited an alignment of chromatin along the centre of the spindle and extrusion of a single polar body containing chromatin.

Parthenogenetic activation and embryo culture

Cumulus-free oocytes were activated for 5 min at room temperature with 5 μ M ionomycin (Sigma) in HEPES SOF and 10% FCS. They were subsequently washed twice in HEPES SOF with 10% FCS and then washed in SOFaaBSA culture medium (i.e. HEPES-free SOF supplemented with 1 mM-L-Glutamine, 8 mg/ml BSA (fatty acid free), 1 \times essential amino acids (Sigma B6766), 1 \times non-essential amino acids (Sigma M7145), pH 7.4, osmolarity 265–275) (Thompson *et al.* 1995, Walker *et al.* 1996). Oocytes were then incubated for 4 h in SOFaaBSA containing 2.5 mM 6-dimethylaminopurine (6-DMAP; Sigma) and 35.5 μ M cycloheximide (Sigma) at 38.5 °C in humidified 5% O₂, 5% CO₂ and 90% N₂ atmosphere. Following activation, oocytes were washed three times in SOFaaBSA and transferred into 4-well Nunc plates for final culture in 500 μ l SOFaaBSA under mineral oil. Embryos were examined for cleavage at 24 h post activation (day 1), and for blastocyst development on day 7. Embryo culture media was supplemented with FCS to a final concentration of 9% on day 5 of culture. Blastocyst cell nuclei were stained by addition of Hoechst 33342 (Sigma) to the culture medium at 5 μ g/ml and incubated for 15 min. Subsequently, embryos were mounted on glass slides and visualised by fluorescence microscopy to determine their nuclear number.

Statistical analysis

The developmental data (proportion of oocytes reaching MII and blastocysts) were analysed by logistic regression. Effects of replicate and treatment, and their interaction were incorporated into the analysis. Also, the treatment effect was split into contrasts between one or two treatments and the rest in order to examine the differences between them. The interaction between treatment and replicate was found to be not significant. Results from all replicates were pooled for presentation. The data on blastocyst nuclei count were analysed by ANOVA to look for treatment and replicate effects.

Results

Evaluation of BDNF and receptor mRNA expression

We first considered whether mRNA for BDNF and associated receptors could be detected in RNA isolated from bovine ovary COCs, and cumulus and oocytes separately. Amplification products of the expected size corresponding to BDNF (198 bp), TrkB (206 bp), truncated TrkB (430 bp) and p75 (153 bp) receptors were first detected in RNA

extracted from bovine ovarian tissue and confirmed by DNA sequencing (data not shown). All gene transcripts were also detected in RNA isolated from COCs, and cumulus cells alone (Fig. 1). Neither full length or truncated TrkB was ever detected in oocytes in six independent trials which simultaneously confirmed their presence in COCs and cumulus mRNA. It is therefore likely that expression in the former was reflective of transcripts in the cumulus cell component. In contrast, both BDNF and p75 could be detected in maturing and MII arrested oocytes sampled 26 h post *in vitro* maturation (IVM) in five independent trials.

Immunohistochemistry for BDNF and associated receptors

We next evaluated whether BDNF and its receptors could be localised in the bovine ovary and COCs by immunohistochemistry. BDNF specific staining was evident in both oocytes and granulosa cells of preantral and antral follicles, and in expanded cumulus and oocytes following IVM (Fig. 2). While BDNF expression in oocyte cytoplasm was only faint in ovarian tissue sections, it was more marked in isolated COCs (Fig. 2C). Cumulus cells also exhibited strong immunostaining. Some immunostaining of the theca cell layer surrounding antral follicles was also evident, as was prominent staining of vascular endothelial cells (Fig. 2B). No staining was detected in negative controls, following preabsorption of the primary antibody with BDNF peptide (Fig. 2D–F).

A similar pattern of distribution as for BDNF was also evident for p75. Thus p75 was expressed by granulosa cells of preantral and antral follicles (Fig. 2G and 2H), with faint staining of theca cells surrounding antral follicles (Fig. 2H). Oocytes of isolated COCs were positive for p75-specific immunohistochemical staining (Fig. 2I). We were unable to obtain clear specific immunostaining for TrkB, although staining was detected in rat brain as a positive control (not shown).

Effect of BDNF on maturation of cumulus-enclosed oocytes to MII

To evaluate the effect of BDNF on bovine oocyte maturation, cumulus-enclosed oocytes were matured in the presence (positive control) or absence (negative control) of 10% FCS, or in serum-free media supplemented with 10 or 100 ng/ml BDNF, 5 μ g/ml anti-BDNF blocking antibody alone, or the latter with 10 ng/ml BDNF. All oocytes in each treatment group had undergone germinal vesicle breakdown (GVBD) by the time they were analysed. In total 77% of COCs in maturation medium containing FCS were scored as MII arrested after 26 h of culture. This was 2–3-fold greater ($P < 0.01$) than that observed in all other treatment groups, between which there was no significant difference in outcome (Fig. 3). Thus, supplementation of serum-free maturation medium with BDNF was insufficient to substitute for the known benefit of serum to

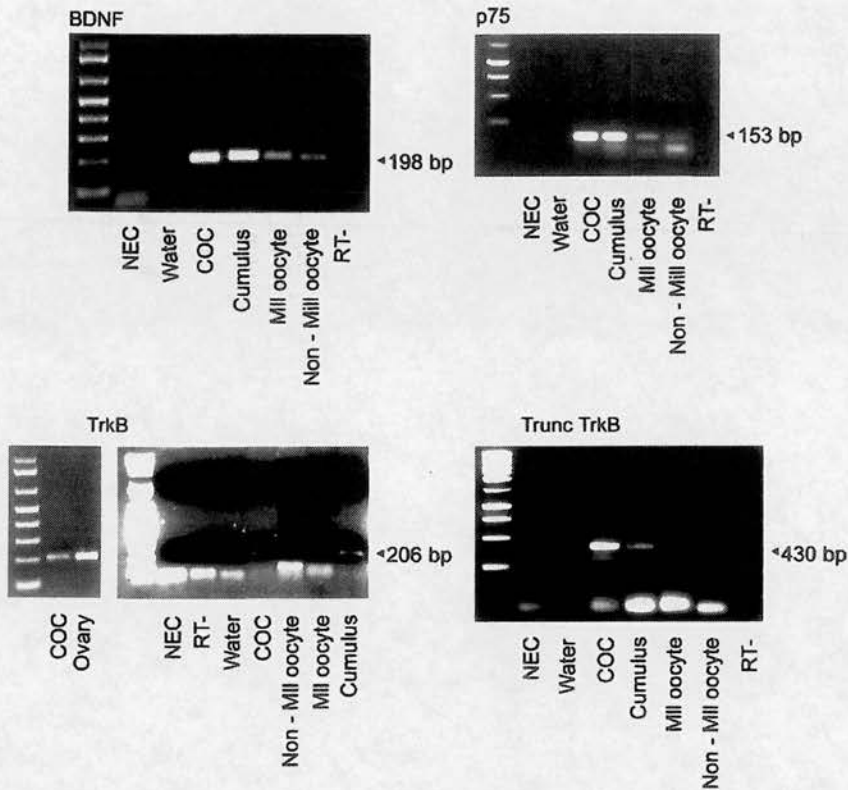


Figure 1 Expression of BDNF, p75 and TrkB mRNA isoforms in bovine cumulus and oocyte complexes. By RT-PCR, cDNA amplification products representative of BDNF (198 bp), p75 (153 bp), TrkB (206 bp) and truncated TrkB (Trunc TrkB, 430 bp) mRNA are detectable in RNA prepared from whole bovine cumulus–oocyte complexes (COC), cumulus alone, or ovary. In mature (MII) and maturing (non-MII) oocytes collected 26 h post *in vitro* maturation, only BDNF and p75 mRNA transcripts could be detected. As negative controls, PCR was performed on water, tissue/cell extract with omission of bulk first-strand cDNA synthesis (RT–) and reagent blank non-embryo control (NEC).

support nuclear maturation, when assessed at a single point in time when oocytes should have reached MII. Furthermore, a blocking antibody to BDNF did not have any additive detrimental effect on nuclear maturation above that observed for serum-free maturation in general.

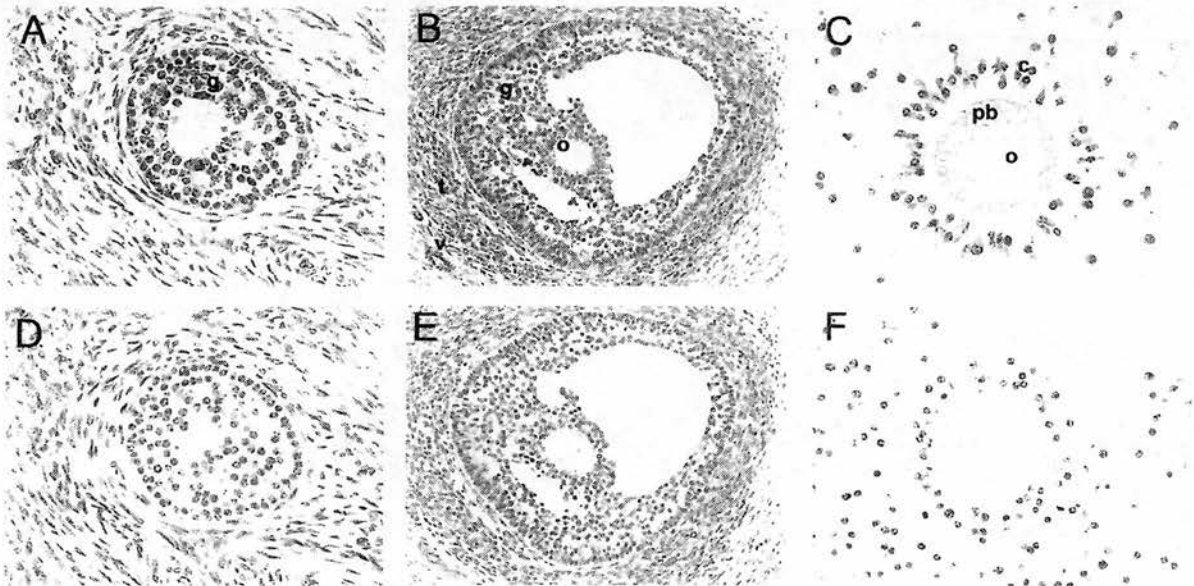
Effect of BDNF during maturation of cumulus-enclosed oocytes on parthenogenetic embryo development

Parthenogenetic embryo development to cleavage and blastocyst stages were evaluated following activation of oocytes matured as COCs in the previously defined treatment groups. In these trials, no attempt was made to score maturation status of oocytes following IVM, thus most oocytes, except those damaged during removal of cumulus, subsequently underwent parthenogenetic activation, irrespective of maturation status. In a first series consisting of three replicate trials, COCs matured without serum and activated (total $n = 83$) were compared with those matured with FCS ($n = 103$) or either 10 ($n = 95$) or 100 ng/ml BDNF ($n = 91$; Fig. 4A–C). Evaluating the proportion of activated oocytes undergoing first cleavage after 24 h, there were no significant differences across treatment groups (serum-free, 63%; FCS, 65%; 10 ng/ml BDNF, 65%; 100 ng/ml BDNF, 74%; Fig. 4A). By contrast, the proportion of activated oocytes forming blastocysts following maturation with 10 ng/ml BDNF was over 1.5-fold higher ($P < 0.01$) than with FCS, which was equivalent to the higher concentration of BDNF. Blastocyst yields in all

treatment groups were also significantly higher than oocytes matured without serum (serum-free, 10%; FCS, 15%; 10 ng/ml BDNF, 24%; 100 ng/ml BDNF, 18%; $P < 0.01$). Improved blastocyst development following maturation with the lower concentration of BDNF was also apparent if calculated relative to the number of cleaved embryos (serum-free, 15%; FCS, 22%; 10 ng/ml BDNF, 37%; 100 ng/ml BDNF, 24%; $P < 0.01$).

In a second series of three replicate trials, the development of COCs matured without serum was compared against those matured with 10 ng/ml BDNF (the best treatment in the previous series), 10 ng/ml BDNF + 5 μ g/ml blocking antibody, or 5 μ g/ml blocking antibody alone. The proportion of activated oocytes undergoing first cleavage after 24 h was significantly lower following maturation with the blocking antibody alone (30%, $n = 81$) vs all of the other treatment groups (i.e. serum-free, 64%, $n = 83$; 10 ng/ml BDNF, 60%, $n = 85$; 10 ng/ml BDNF + blocking antibody, 63%, $n = 76$; $P < 0.01$). Blastocyst yields in this experimental series were generally poorer than that observed in the first series, possibly due to seasonal variations in oocyte quality commonly encountered with abattoir derived ovaries. Despite this, treatment with 10 ng/ml BDNF yielded significantly more blastocysts per activated oocytes vs any of the other treatment groups (10 ng/ml BDNF, 9%; serum-free, 2%; BDNF + blocking antibody, 4%; blocking antibody alone, 4%; $P < 0.01$). Evaluating blastocyst nuclear counts for each treatment

BDNF



p75

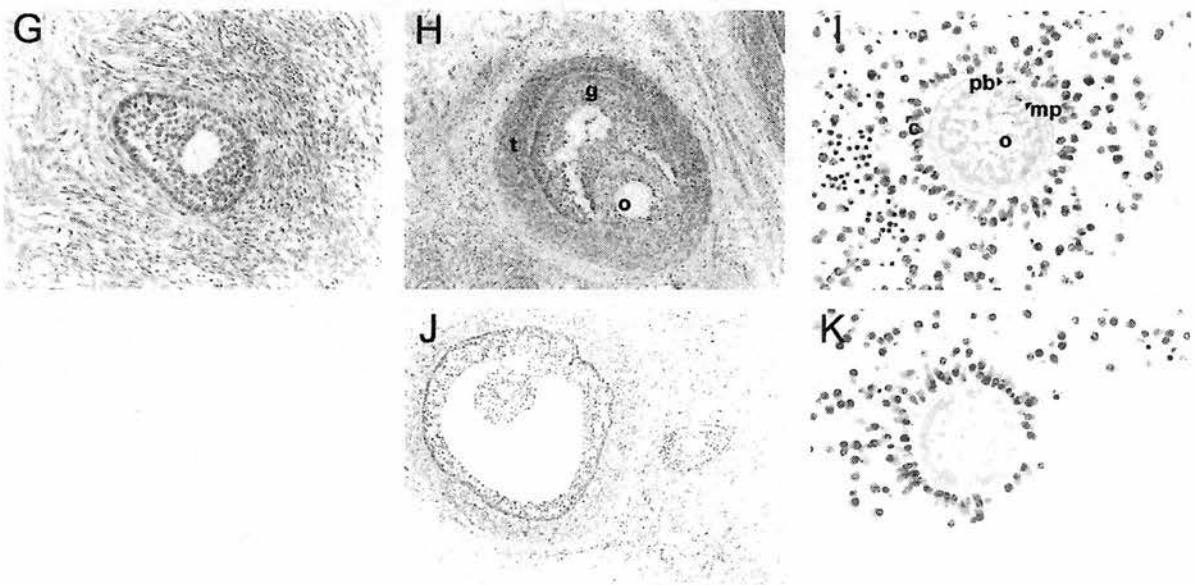


Figure 2 Immunohistochemical staining for BDNF and p75 in bovine ovarian follicles and cumulus oocyte complexes (COCs). BDNF specific immunostaining was evident in both oocytes (o) and granulosa (g) cells of preantral (A) and antral (B) follicles and in cumulus (c) and oocytes following *in vitro* maturation of isolated COCs (C). Some immunostaining of the theca (t) cell layer surrounding antral follicles was also evident, as was prominent staining of vascular (v) endothelial cells. In matched serial sections, this staining was blocked (D–F) if the primary antibody was first pre-incubated with the BDNF blocking peptide. Immunostaining for p75 (G–I) which was above that observed following omission of the primary antibody (J, H), was also detected in the granulosa and thecal cell layer of preantral (G) and antral follicles (H), and in oocytes and cumulus cells of *in vitro* matured COCs (I, J). Polar bodies (pb) and a metaphase plate (mp) are labelled.

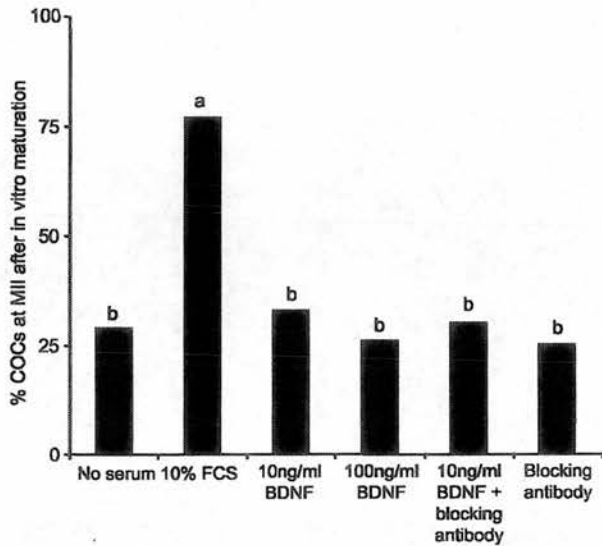


Figure 3 Effect of BDNF on maturation of cumulus-enclosed oocytes to MII. COCs were matured in the presence (positive control) or absence (negative control) of 10% FCS, or in serum-free media supplemented with 10 or 100 ng/ml BDNF, 5 µg/ml anti-BDNF blocking antibody alone, or the latter with 10 ng/ml BDNF. The experiment was repeated four times to arrive at the average proportion of oocytes at MII after 26 h of culture. Different letters denote significant differences ($P < 0.01$). Number of COCs per group: serum-free $n = 49$; 10% FCS $n = 43$; 10 ng/ml BDNF, $n = 49$; 100 ng/ml BDNF, $n = 47$; 5 µg/ml anti-BDNF, $n = 48$; 10 ng/ml BDNF + 5 µg/ml anti-BDNF, $n = 47$.

within and between experiment trials revealed no significant differences (i.e. for each treatment group, means \pm S.E.M., serum-free, 68 ± 8 , $n = 12$; FCS, 68 ± 5 , $n = 15$; 10 ng/ml BDNF, 65 ± 6 , $n = 31$; 100 ng/ml BDNF, 77 ± 6 , $n = 16$; 10 ng/ml BDNF + blocking antibody, 54 ± 6 , $n = 3$; blocking antibody alone, 63 ± 10 , $n = 3$).

Effect of BDNF on maturation of cumulus-free oocytes to MII

To evaluate the effect of BDNF on bovine oocyte maturation without cumulus, germinal vesicle oocytes were denuded at the time of collection and matured in medium with or without 10% FCS, or in serum-free media supplemented with 10 ng/ml BDNF, 10 ng/ml BDNF + 5 µg/ml anti-BDNF blocking antibody, or 5 µg/ml anti-BDNF blocking antibody alone. These were compared against cumulus enclosed oocytes matured with 10% FCS (positive control). At the time of analysis all oocytes had undergone GVBD. Similar to the first maturation experiment evaluating BDNF on cumulus enclosed oocytes, 71% ($n = 73$) of COCs matured with FCS were scored as MII arrested after 26 h of culture. This was significantly higher than cumulus-free oocytes matured with FCS (39%, $n = 69$; $P < 0.01$), which in turn was approximately double ($P < 0.01$) that observed in all of the other treatment groups (i.e. serum-free, 22%, $n = 69$; 10 ng/ml

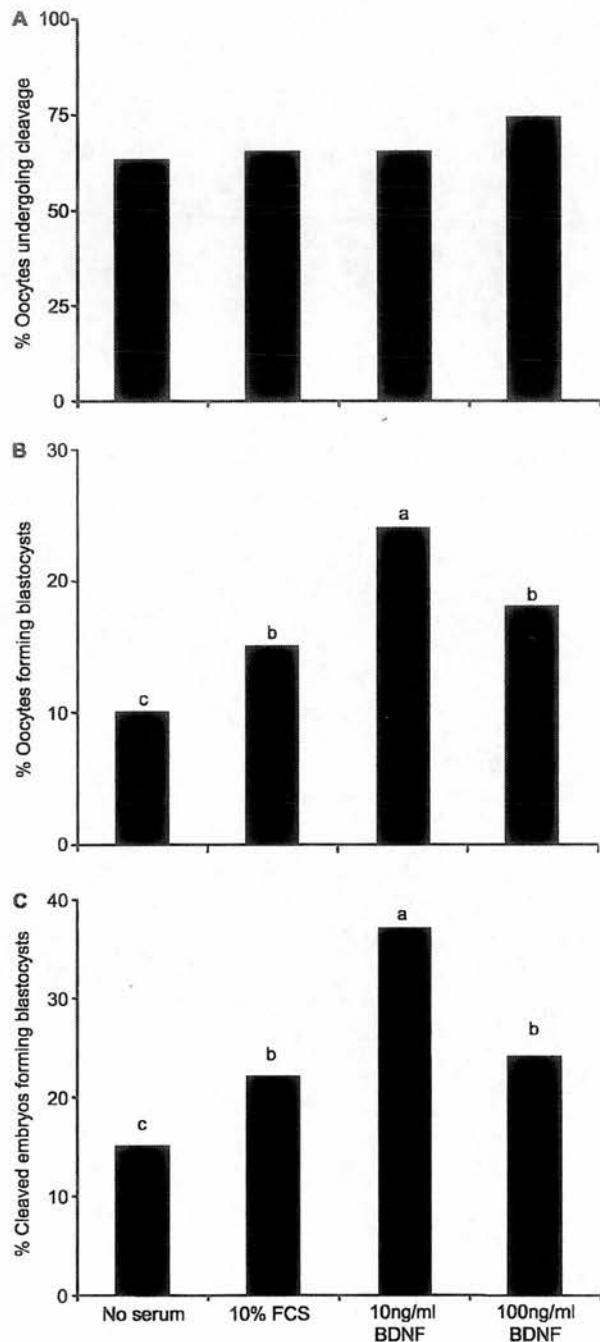


Figure 4 Development of parthenogenetic embryos following *in vitro* maturation of cumulus-enclosed oocytes with BDNF. COCs were matured in the presence (positive control) or absence (negative control) of 10% FCS, or in serum-free media supplemented with 10 ng/ml or 100 ng/ml BDNF, then activated. Data from three replicate trials were pooled to arrive at the average proportion of activated eggs undergoing cleavage (A) or forming blastocysts (B), or the proportion of cleaved embryos forming blastocysts (C). Different letters (a–b) denote significant differences ($P < 0.01$). Number of activated oocytes per group: +FCS, $n = 103$; serum-free, $n = 83$; 10 ng/ml BDNF, $n = 95$; 100 ng/ml BDNF, $n = 91$.

BDNF, 25%, $n = 71$; BDNF + blocking antibody, 19%, $n = 36$; and blocking antibody alone, 18%, $n = 49$). Thus, although progression to MII is generally poorer in the absence of cumulus cells, exogenous BDNF cannot substitute for serum to support nuclear maturation in the presence or absence of cumulus cells. Furthermore, a blocking antibody to BDNF did not have any additive detrimental effect on nuclear maturation beyond that observed for serum-free maturation in general.

Effect of BDNF during maturation of cumulus-free oocytes on parthenogenetic embryo development

In a final series of experiments, parthenogenetic embryo development to cleavage and blastocyst stages was evaluated following activation of oocytes matured in the absence of cumulus cells as described above. As with the activation experiments involving cumulus-enclosed maturation, the maturation status of oocytes was not scored and all morphologically intact oocytes were activated. This design was repeated in 5 replicate trials, each of which also included a positive control of COCs matured with 10% FCS (Fig. 5A–C). The proportion of activated oocytes that had cleaved by 24 h was significantly higher for COCs and stripped oocytes matured with FCS and stripped oocytes matured with BDNF, vs all of the other treatment groups (i.e. COCs + FCS, 61%, $n = 114$; stripped + FCS, 53%, $n = 119$; stripped serum-free, 32%, $n = 115$; 10 ng/ml BDNF, 43%, $n = 116$; 10 ng/ml BDNF + blocking antibody, 29%, $n = 118$; blocking antibody alone, 28%, $n = 120$; $P < 0.05$). Similarly, the proportion of activated oocytes forming blastocysts was significantly highest for COCs matured with FCS (33%). Cumulus-free oocytes matured with serum (13%) or 10 ng/ml BDNF (7%) were in turn significantly different from BDNF with blocking antibody (4%), and serum-free (2%), with no blastocysts formed by treatment with blocking antibody alone ($P < 0.01$). A similar relationship between treatment groups was observed if blastocyst yields were expressed relative to numbers of cleaved embryos ($P < 0.01$), with the exception that there no longer was a significant difference between BDNF and BDNF with blocking antibody. Irrespective of differences in the proportion of embryos forming blastocysts there were no differences in blastocyst nuclear counts between cumulus-free treatment groups (means \pm S.E.M.: stripped + FCS, 61 ± 4 , $n = 15$; stripped serum-free, 53 ± 13 , $n = 2$; 10 ng/ml BDNF, 57 ± 6 , $n = 8$; 10 ng/ml BDNF + blocking antibody, 59 ± 5 , $n = 5$). However, blastocyst nuclear counts for COCs matured in medium containing serum were significantly higher than cumulus-free groups (85 ± 8 , $n = 38$; $P < 0.01$).

These findings suggest that BDNF can substitute for serum but not cumulus cells during IVM in order to support oocyte competence to form parthenogenetic blastocysts. The capacity of IVM treatment with blocking

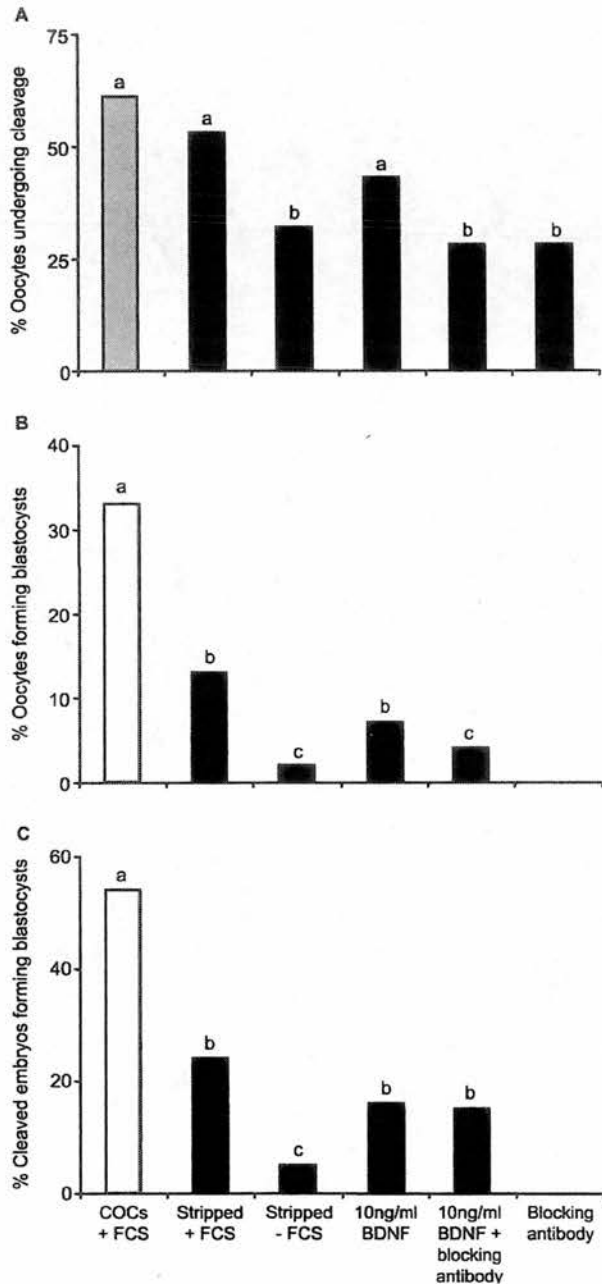


Figure 5 Development of parthenogenetic embryos following *in vitro* maturation of cumulus-free oocytes with BDNF or blocking antibody. Germinal vesicle oocytes were denuded at the time of collection and matured in medium with or without 10% FCS (serum), or in serum-free media supplemented with 10 ng/ml BDNF, 10 ng/ml BDNF + BDNF blocking antibody, or blocking antibody alone prior to activation. Data from five replicate trials were pooled to arrive at the average proportion of activated eggs undergoing cleavage (A) or forming blastocysts (B), or the proportion of cleaved embryos forming blastocysts (C). Significant differences denoted by different letters (a–c; $P < 0.01$). Number of activated oocytes per group: COCs + FCS, $n = 114$; stripped oocytes + FCS, $n = 119$; stripped oocytes – FCS, $n = 115$; 10 ng/ml BDNF, $n = 116$; 10 ng/ml BDNF with blocking antibody, $n = 118$; blocking antibody alone, $n = 120$.

antibody to completely block blastocyst formation suggests that BDNF may also exert an influence on oocytes directly.

Discussion

Using a bovine oocyte IVM and parthenogenesis model system we have investigated the role of BDNF in conferring oocyte developmental competence. Both oocytes and cumulus express mRNA transcripts and protein for BDNF and the p75 receptor that has affinity for all neurotrophins, but transcripts for the full length and truncated forms of the specific TrkB receptor are confined to cumulus. BDNF cannot substitute for serum in supporting meiotic progression to MII during IVM of cumulus-enclosed or -free oocytes. Despite this it can increase or approach the yield of parthenogenetic blastocysts relative to serum mediated maturation of cumulus-enclosed or -free oocytes, respectively, via signalling pathways which are sensitive to a BDNF specific blocking antibody. These results suggest that BDNF plays a role in conferring oocyte cytoplasmic competence to support early embryo development, independently of nuclear maturation, and that this may involve both autocrine and paracrine signalling within COCs.

Our study relied on parthenogenesis rather than fertilisation to assess oocyte competence for embryonic development. In mammals parthenogenesis can yield viable offspring provided that parent-specific imprints regulating gene expression are overcome to permit the formation of a functional placenta (Kono *et al.* 2004). Without such manipulations, parthenogenesis still provides a valuable measure of oocyte competence to initiate a developmental program since development to the blastocyst stage is independent of epigenetic imprinting (Latham *et al.* 1994). Accordingly, it is commonly used to assess oocyte competence to support early development following somatic or pronuclear transfer (Liu *et al.* 2001, De Sousa *et al.* 2002). The method of parthenogenetic activation used in our study has previously been characterised to yield equivalent rates of development to the blastocyst stage in a direct comparison with fertilised oocyte cohorts, although with a significant reduction in resulting cell number, similar to the present results (De La Fuente & King 1998).

Important roles for Trk B receptors in oocyte survival in both the perinatal period of primordial follicle formation and during the early stages of follicle growth have been demonstrated using mouse knockout models (Spears *et al.* 2003, Paredes *et al.* 2004). Unfortunately however, these models have failed to illuminate the roles for these receptors or BDNF in the later stages of ovarian follicle development and for oocyte competence, due to the neonatal lethality that they invoke (Klein *et al.* 1993, Ernfors *et al.* 1994). BDNF, NT-4 and NT-3 have all been detected in adult human follicular fluid (Seifer *et al.* 2002b, Seifer *et al.* 2003). *In vitro* studies on human cumulus cells have also found that BDNF secretion can be stimulated by treatment with cAMP and human chorionic gonadotropin

(hCG), but not recombinant FSH (Feng *et al.* 2003). Both BDNF and NT-4, but not NT-3 also promoted first polar body extrusion in cumulus-free mouse oocytes matured *in vitro*, compared with those matured in the absence of serum or hormones (Seifer *et al.* 2002a,b). Accordingly, by immunohistochemistry these studies have reported that most mouse oocytes are positive for TrkB, the BDNF and NT-4 receptor tyrosine kinase, with no immunoreactivity for TrkC, the respective receptor for NT-3. By RT-PCR the present data show p75 but not TrkB isoform mRNAs in bovine oocytes. Furthermore, we found no difference between BDNF and serum-free treatment groups in the proportion of oocytes reaching MII, both of which were inferior to supplementation with serum. This may reflect a species difference.

Our current study provides the first evidence that BDNF may benefit oocyte competence for embryonic development without necessarily benefiting maturation. Whereas approximately 80% of oocytes matured with FCS (positive control) reached MII, this was reduced to 30% in all FCS-free experimental treatments, including those with BDNF. In succeeding trials there was no difference between BDNF and FCS treatment groups in the proportion of parthenogenetic embryos which cleaved (~60%), with blastocyst yields improved or matched by BDNF treatment. Serum-free IVM has previously been shown to yield significantly fewer oocytes reaching MII, 50–60% vs 80–90% with serum (Loneragan *et al.* 1994, Ali & Sirard 2002). Our serum-free MII yields were generally lower. If in the absence of serum BDNF is inhibitory to meiotic maturation, this would not have been apparent in our experiments. However there was no evidence of an inhibitory effect of BDNF on subsequent parthenogenetic development.

Cumulatively, our results indicate a role for BDNF in oocyte maturation enabling both early embryo cleavage and blastocyst formation. For both cumulus-enclosed and -free maturation, the effect of the BDNF blocking antibody was first manifested by a significant reduction in cleavage. Compared with the serum treatment group, the improvement or matching of blastocyst yields with exogenously supplied BDNF was not paralleled by improved cleavage. Since BDNF did not increase the quantity of cells in blastocysts, it is likely that its effect was on oocyte and embryo survival as opposed to the promotion of growth. Further work is necessary to determine if BDNF has a physiological role during oocyte maturation.

BDNF signalling between cumulus and oocytes may be bi-directional with functionally different consequences given that both cell types express BDNF and differentially express p75 and TrkB isoforms, namely oocytes lack the latter. Trk and p75 receptors do not bind directly to each other, but can be complexed together (Bibel *et al.* 1999, Lee *et al.* 2001). This allows the signalling pathways triggered by both receptors to interact. The association of the two receptor types results in higher affinity ligand binding and a greater discrimination between neurotrophins. Thus,

BDNF, NT3 and NT4 can each bind to the TrkB receptor, but in the presence of p75, only BDNF provides a functional response (Bibel *et al.* 1999). In neurons, Trk receptors and their substrates can activate three main signalling cascades: 1) differentiation, via a Ras/Raf/ MEK/MAP kinase pathway; 2) cell survival (anti-apoptosis) by association with insulin receptor substrates leading to inactivation of proapoptotic proteins; and 3) calcium release from internal stores via PLC- γ mediated production of IP₃ and production of protein kinase C, which in neurons plays a role in neurotrophin mediated neurotrophin release (for review see Bibel & Barde 2000). All of these pathways may be relevant to follicular and oocyte maturation, but most striking is the potential for neurotrophin mediated stimulation of the MAP kinase pathway. Gonadotropin induced cumulus expansion and resumption of meiosis in oocytes is dependent upon activation of MAP kinase in granulosa cells. This activation is downstream of gonadotropin-induced elevation of granulosa cell cAMP, and is dependent upon one or more paracrine factors from the oocyte (Su *et al.* 2003). BDNF secretion by cumulus is another consequence of either gonadotropin stimulation or artificial elevation of cAMP. Elevated cAMP in cumulus could lead to the same in oocytes via gap junctions, culminating in BDNF secretion by oocytes that could act in an autocrine or paracrine fashion to augment the effect of cumulus-derived BDNF. It is unknown whether BDNF derived from oocytes or cumulus would differ in their bioactivity. Neurotrophins in general are substrates for pro-protein convertases that can alter the molecular bioactivity of their targets by proteolytic cleavage during intracellular and extracellular processing. BDNF is an especially well characterised example of convertase-dependent modulation of bioactivity (reviewed in Seidah & Chretien 1999). Thus, differences in BDNF bioactivity could be achieved by differential expression of pro-protein convertases in cumulus and oocytes.

The functional consequences of ligand interaction with the p75 receptor are also complex, and in neurons have been linked to cell survival, arrest, differentiation, and programmed cell death. Recently the concept of p75 as a 'quality control' receptor has been advanced based on evidence that it is capable of mediating programmed cell death in response to either ligand binding or withdrawal. In essence the downstream consequence of ligand interactions are proposed to be dependent on the relative proportions of p75 and Trk receptors and which neurotrophin is present (Rabizadeh & Bredesen 2003). According to this model, binding of neurotrophins to p75 in the absence of Trk receptors, typically suppresses apoptosis. This could be the case for bovine oocytes provided they do not express TrkA or TrkC receptors. From mouse knockout models, mice homozygous for a targeted mutation in p75, rendering it functionally inactive, are viable and fertile, although they eventually develop deficits in their peripheral sensory nerves characterised by heat sensitivity and susceptibility to ulceration (Lee *et al.* 1994). This suggests

that the most critical role for p75 may be to ameliorate the effect of stress, which in the case of oocyte maturation and developmental competence, might be most affected by adverse *in vitro* culture environments.

A number of other growth factors have been reported to improve oocyte competence to support embryo development, when applied during oocyte maturation *in vitro*. Epidermal growth factor (EGF) improves the yield of fertilised bovine blastocysts, but not cell number, relative to serum-free culture conditions lacking hormones (Loneragan *et al.* 1996). However, this effect is preceded by promotion of the proportion of cumulus-enclosed or -free oocytes progressing to MII, the latter supporting a direct effect on oocytes. EGF-receptor tyrosine kinase mediated activation of meiosis was first described in *Xenopus* oocytes, and later confirmed in the rat and mouse (Maller 1985, Ueno *et al.* 1988, Downs 1989). Studies in the latter also revealed a direct effect of EGF on cumulus, leading to the promotion of cumulus expansion. Like EGF, other groups have demonstrated that supplementation of bovine oocyte IVM culture with exogenous activin A, promotes meiotic maturation and subsequent yield of fertilised blastocysts (Stock *et al.* 1997, Silva & Knight 1998), an effect which is reduced by the activin binding protein follistatin. These gene products are synthesised by granulosa cells and are prominent components of follicular fluid (Braw-Tal 1994). Activin A receptor mRNA can be detected in both granulosa cells and oocytes (Cameron *et al.* 1994). In the former, activin A appears to increase the number of FSH receptors in granulosa cells and as such may promote gonadotropin action during maturation (Nakamura *et al.* 1993). A direct effect of activin A on oocytes is supported by evidence of improved progression to MII of cumulus-free oocytes, although the mechanism by which this is achieved is unknown (Stock *et al.* 1997). Activin A also promotes human germ cell survival and proliferation prior to primordial follicle formation (Martins da Silva *et al.* 2004). Only midkine, a heparin-binding growth/differentiation factor, which is also a prominent feature of bovine follicular fluid, is similar to BDNF in promoting oocyte competence to reach the blastocyst stage without also improving meiotic progression to MII (Ikeda *et al.* 2000). Midkine is also produced by granulosa cells under the control of gonadotropins (Minegishi *et al.* 1996). Unlike BDNF however, midkine does not appear to act directly on oocytes. Its actions can also be blocked by heparin (Ikeda *et al.* 2000).

In conclusion, our study adds BDNF to the list of growth factors, normally present in follicular fluid, which can specifically promote oocyte developmental competence during IVM. BDNF differs from most other factors identified however in that its effect is to promote cytoplasmic maturation without advancing nuclear maturation. This effect is likely to be complex and mediated by direct effects on both the oocyte and cumulus cells. This knowledge will be of value to the creation of completely defined culture environments free of serum for oocyte maturation,

which will improve both their overall safety and the efficacy of assisted reproductive technologies applied to both animals and humans.

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Neurotrophin Signaling in Oocyte Survival and Developmental Competence: A Paradigm for Cellular Toti-Potency

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ABSTRACT

While not fulfilling the criterion of a "stem cell" in being capable of self-renewal, mature and fertilized oocytes are the original "toti-potent" cells, whose capacity for expansion and differentiation can only be approximated by stem cells of embryonic or adult origin *in vitro*. As such, the mechanisms by which oocytes acquire and manifest competence to support embryo development is of fundamental interest to efforts to control and re-specify somatic cell fate and toti-potency. This is underscored by the unparalleled capacity of oocyte cytoplasm to successfully re-specify the genetic program of animal development following cell nuclear replacement (i.e., cloning). Thus, the knowledge gained by understanding the acquisition of oocyte developmental competence could ultimately facilitate the creation of adult stem cells *in vitro* from terminally differentiated cells, *ex ovo*. In this paper, we review the concept of oocyte developmental competence, and focus on our own research and that of others implicating a role for neurotrophins in this process, and that of oocyte cell survival. Lastly we propose a role for neurotrophin signalling in embryo stem cell survival.

INTRODUCTION

OOCYTE COMPETENCE to support embryo development is acquired gradually over a prolonged period of oocyte growth (i.e., oogenesis) and follicular development (i.e., folliculogenesis), and is reliant on complex bi-directional interactions between germ and follicular somatic cells beginning in the fetus and extending into adult life (Buccione et al., 1990; Eppig, 2001). While initial follicular formation and growth is regulated by intraovarian factors (Matzuk et al., 2002), later development and final maturation are under the overall control of pituitary gonadotropins. In fetal ovaries, mitotically dividing female germ cells (i.e., oogonia) become oocytes as they initiate meiosis and arrest at the diplotene stage of

prophase I. This occurs as they become surrounded by a flattened layer of somatic pregranulosa to form primordial follicles. Also concurrent with this process is a massive wave of oocyte cell death that results in the loss of up to 90% of oocytes in mice and humans (Hirshfield, 1991). This event determines female reproductive lifespan by limiting the supply of primordial follicles from which developmentally competent oocytes can be derived in the future. Its timing in mammals can vary from mid-gestation (5 months) in humans to around the time of birth in mice.

Oocytes within primordial follicles are regarded as being in a resting state, and competence to support embryo development is only acquired after the completion of oocyte growth and maturation. Oocyte growth commences with the

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formation of primary follicles, distinguished from primordial follicles by the rounding of the single layer of granulosa cells adhering to oocytes. Granulosa cells continue to proliferate into several layers and subsequent antrum formation is correlated with oocyte competence to undergo meiotic maturation. Oocyte growth is typified by a prolonged phase of intensive RNA and protein synthesis and storage which is accompanied late in oogenesis by epigenetic modifications of oocyte chromatin that are necessary for regulating gene expression and development post-implantation (Kono et al., 1996). Transcription ceases once oocytes undergo maturation and resume meiosis, which *in vivo* is triggered by an ovulation-inducing surge of luteinizing hormone (LH). In the mouse, oocyte transcriptional silencing in antral follicles has been shown to correlate with chromatin remodeling and is modulated by granulosa cell interactions that precede resumption of meiosis (De La Fuente et al., 2001).

Oocyte maturation is characterized by both nuclear and cytoplasmic changes. The former involves nuclear envelope breakdown and the meiotic segregation of chromatin to yield a haploid set of replicated chromosomes, whose final segregation awaits fertilization or an activation stimulus. In contrast to this fairly defined process, the latter more broadly entails the acquisition of competence to initiate a cascade of developmental events. This includes the capacity to (1) respond to signal transduction triggered by autocrine, paracrine, or endocrine stimulation, and sperm or artificial activation; (2) structurally remodel sperm and oocyte chromatin and nuclei to form a new functionally integrated genome that will divide mitotically; and (3) transcriptionally activate and regulate the expression of the newly formed genome to specify a developmental program. The complexity and interrelation of these capacities has made cytoplasmic maturation comparatively poorly understood.

During oocyte maturation, gene expression is regulated by selective mRNA recruitment. This involves mRNA sub-localization, translation, adenylation, and degradation, as well as post-translation modification of newly synthesised and existing proteins (Eichenlaub-Ritter et al., 2002). Competence to resume and complete meiosis during maturation and in response to an activation stimulus is associated with an accumulation of cell cycle regulatory molecules, such as for

example an increase in the nuclear concentration of both cyclin B and p34^{cdc2} (Albertini et al., 1998; Mitra et al., 1996). Other factors that undergo dynamic shifts between cytoplasmic and nuclear compartments during oocyte growth and maturation include those involved in the regulation of genomic imprinting, calcium signalling, and microtubules spindle organization (Avazeri et al., 2000; Combelles et al., 2001; Howell et al., 2001).

While meiotic and cytoplasmic maturation are both necessary, the former does not ensure or guarantee the latter. Thus, in mice, *in vitro* oocyte maturation in the absence of cumulus cells yields oocytes that can complete meiotic segregation of chromatin and even pronuclear formation after fertilization or activation; however, the chromatin assembled in pronuclei by the cytoplasm of such oocytes has almost no competence to support development of resulting offspring (Liu et al., 2001). Errors in meiotic recombination that can be induced by mutations in gene products required for this process do not block oocyte nuclear maturation, although they do block spermatogenesis (Hunt et al., 2002). Although these errors can result in aneuploid eggs, they do not necessarily result in embryonic lethality, as evidenced in humans by non-dysjunction of chromosome 21 resulting in Down's syndrome.

Oocyte cytoplasmic competence is likely to be dependent on genes encoding factors involved in chromatin remodeling, such as those associated with the SWI/SNF remodelling complex. Embryos lacking components encoded by SNF5 or BRG1 genes develop normally to the blastocyst stage, but die in the perimplantation period (LeGouy et al., 1998; Klochendler-Yeivin et al., 2000). In amphibian oocytes, nucleoplasmin is known to remodel sperm chromatin or transplanted somatic nuclei in the absence of DNA replication (Brown et al., 1987; Gurdon et al., 1976). However, the role of mammalian homologs is not as well understood. Female mice with a null mutation for one homolog, mNPM2 are infertile, despite the fact that decondensation of sperm nuclei occurs, with severe abnormalities apparent in the nucleolar organization of oocytes and embryos (Burns et al., 2003). Other proteins that are able to assemble nucleosomes in the absence of DNA replication include nucleosome assembly protein 1 (NAP-1) (Ishimi et al., 1991), which is known to be expressed in mouse oocytes (McLay et al., 2002).

GROWTH FACTORS CONFERRING OOCYTE DEVELOPMENTAL COMPETENCE DURING MATURATION

Culture systems supporting oocyte and follicular growth and oocyte maturation remain the subject of intensive research across a diverse range of species. However, the generation of developmentally competent oocytes from primordial follicles *in vitro* is an achievement that to date has only been possible in the mouse (O'Brien et al., 2003; Thomas et al., 2003). By contrast *in vitro* systems for the maturation of full-grown oocytes from antral follicles exist in numerous species, with the most notable success observed in the bovine. While variations exist across species, the developmental potential of oocytes in these systems has generally benefited by the supplementation of culture media with follicular fluid or serum, specific gonadotrophins, steroid hormones, retinoids, and different energy substrates and nutrients. These have been found to benefit oocytes both directly or via cumulus cells (Chian et al., 2004; Sutton et al., 2003). Specific growth factors identified as intra-ovarian regulators of oocyte maturation, shown to be beneficial in one or more animal models of oocyte maturation include epidermal growth factor (EGF), insulin-like growth factor I (IGF-I), activin A, inhibin A, and Midkine, a heparin-binding growth factor (Downs, 1989; Eppig et al., 1998; Ikeda et al., 2000; Loneragan et al., 1996; Rieger et al., 1998; Sadatsuki et al., 1993; Silva et al., 1998; Stock et al., 1997; Wang et al., 1995).

The targets and effects of growth factors during oocyte maturation *in vitro* can be distinct as evidenced by studies in the bovine (Table 1). For example, when applied during oocyte maturation culture, EGF improves the subsequent yield of fertilized bovine blastocysts, but not cell number,

when compared with serum-free culture conditions lacking hormones (Loneragan et al., 1996). This effect is preceded by promotion of the proportion of cumulus enclosed or free oocytes progressing to MII, the latter supporting a direct effect on oocytes. EGF-receptor tyrosine kinase mediated activation of meiosis was first described in *Xenopus* oocytes, and later confirmed in the rat and mouse (Downs, 1989; Maller, 1985; Ueno et al., 1988). Studies in the latter also revealed a direct effect of EGF on cumulus leading to the promotion of cumulus expansion. Like EGF, the supplementation of oocyte *in vitro* maturation culture with exogenous activin A promotes meiotic maturation and subsequent yield of fertilized blastocysts (Silva et al., 1998; Stock et al., 1997), an effect that is reduced by the activin binding protein follistatin (Silva et al., 1998). These gene products are synthesised by granulosa cells and are prominent components of follicular fluid (Braw-Tal, 1994). Activin A receptor mRNA can be detected in both granulosa cells and oocytes (Cameron et al., 1994). In the former, activin A appears to increase the number of follicle stimulating hormone (FSH) receptors in granulosa cells and as such may promote gonadotropin action during maturation (Nakamura et al., 1993). A direct effect of activin A on oocytes is supported by evidence of improved progression to MII of cumulus-free oocytes, although the mechanism by which this is achieved is unknown (Stock et al., 1997). In contrast to EGF and activin, midkine, a heparin-binding growth/differentiation factor that is also a prominent feature of bovine follicular fluid, promotes oocyte competence to reach the blastocyst stage without also improving meiotic progression to MII (Ikeda et al., 2000). Midkine is normally produced by granulosa cells under the control of gonadotropins and does not appear to act directly on oocytes (Minegishi et al., 1996).

TABLE 1. EFFECT OF EXOGENOUS GROWTH FACTORS ON *IN VITRO* MATURATION OF BOVINE OOCYTES RELATIVE TO DEFINED SERUM-FREE CULTURE

| Growth factor | MI | Cumulus expansion | Blastocyst | Blastocyst cell number | Target | Reference |
|-------------------|----|-------------------|------------|------------------------|----------|-----------|
| EGF +/- IGF | ↑ | ↑ | ↑ (F) | — | Oo & Cum | a |
| Activin/(Inhibin) | ↑ | ↑ | ↑ (F) | ? | Oo & Cum | b,c |
| Midkine | — | ? | ↑ (F) | ? | Cum | d |
| BDNF | — | — | ↑ (P) | — | Oo & Cum | e |

Fertilised (F). Parthenogenetic (P). Not described (?). No effect (—). Improvement (↑). Oocyte (Oo). Cumulus (Cum). References: ^aLoneragan et al., 1996; ^bSilva et al., 1998; ^cStock et al., 1997; ^dIkeda et al., 2000; ^eMartins da Silva et al., submitted.

NEUROTROPHINS IN OVARIAN FOLLICLE DEVELOPMENT AND OOCYTE SURVIVAL

Neurotrophins are a family of related growth factors initially identified to be important for regulation of neuronal survival and differentiation, but which have also been described in a variety of non-neuronal tissues including the cardiovascular, immune, endocrine and reproductive systems (Matsuda et al., 1988; Polak et al., 1993). They include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophins 3 and 4 (NT3 and NT4). Neurotrophins are unique in that they utilize 2 different receptors to mediate their biological actions: tyrosine kinase (Trk) receptors encoded by the *trk* proto-oncogene family (Trk A-C and truncated isoforms), and the p75 receptor, a member of the tumour necrosis factor (TNF) receptor superfamily (Bibel et al., 2000; Rabizadeh et al., 2003). The p75 receptor is widely expressed and binds all neurotrophins. By contrast, the Trk receptors show selective affinity for different neurotrophins (i.e., TrkA for NGF, TrkB for BDNF, and NT4 and TrkC for NT3). Splice variants and truncated isoforms of Trk receptors lacking intracellular tyrosine kinase domains have also been identified, which retain the capacity to initiate intracellular signalling (Baxter et al., 1997; Bibel et al., 2000).

Intra-ovarian factors contributing to follicular development include oocyte derived growth differentiation factor 9 (GDF-9) and basic fibroblast growth factor (bFGF) and granulosa cell derived Kit-Ligand (Dong et al., 1996; Huang et al., 1993; Nilsson et al., 2001; Matzuk et al., 2002). However, a role for neurotrophins in ovarian development and function, has been suspected since the detection of virtually all ligands and receptors in fetal and adult rodent ovaries (Dissen et al., 1995; Klein et al., 1989; Lamballe et al., 1991). Subsequently, mouse knockout models have suggested roles in early follicular formation and oocyte survival. Mice carrying a null mutation of the NGF gene are deficient in primordial to primary follicle differentiation and subsequent growth (Dissen et al., 2001). Different phenotypes have been described for mice lacking either just full-length or both full-length and truncated TrkB isoforms, the former showing near complete loss of oocytes at the time of primordial follicle formation (Spears et al., 2003), whereas mice lacking all Trk B isoforms are deficient in the growth of

primary follicles, specifically granulosa cell proliferation and differentiation (Paredes et al., 2004). In the mouse, both full length and truncated isoforms of the Trk B (i.e., receptors for BDNF and NT4) are expressed in oocytes in primordial and primary follicles in neonatal ovaries. However, whereas the full length isoform is continually expressed at low levels in oocytes and granulosa cells, the truncated isoform becomes abundantly expressed in oocytes and targeted to the membrane when primary follicles initiate growth (Paredes et al., 2004). Co-incident with this NT4 expression in humans and rodents shifts from germ cells to granulosa cells as mitotically dividing oogonia become oocytes with the formation of primordial follicles (Anderson et al., 2002; Dissen et al., 1995; Paredes et al., 2004). The same has been shown for BDNF in the mouse, and mice carrying null mutations for both BDNF and NT4, but not NT-4 alone, have a similar deficiency in the number of growing secondary follicles as the Trk B isoform null mutants (Paredes et al., 2004).

Without Trk B, oocytes in primary follicles eventually undergo apoptosis, as observed following transplantation of Trk B $-/-$ ovaries into wild type mice (Paredes et al., 2004). Similarly culture of mouse neonatal and human fetal ovaries with a potent inhibitor of all Trk receptors, or alternatively NT-4 and BDNF blocking antibodies, shows that oocyte survival is dependent on Trk B ligands (Spears et al., 2003). Other growth factor signalling is also likely to be important in oocyte survival around the time of follicle assembly, as evidenced by recent studies from some of the authors (S.M. da Silva and R.A. Anderson, 2004, unpublished data) implicating Activin A in the promotion of human oocyte survival and proliferation (Martins da Silva et al., 2004).

NEUROTROPHINS IN OVULATION AND OOCYTE MATURATION AND DEVELOPMENTAL COMPETENCE

Multiple neurotrophins ligands and receptors have also been implicated in the control of ovulation in the adult and more recently, oocyte maturation and acquisition of developmental competence. Increased expression of NGF/TrkA by granulosa cells is induced by the surge in luteinizing hormone (LH) during first ovulation (Dissen et al., 1996), and this is believed to be involved in

the regulation of intercellular gap junction integrity between theca cells (Mayerhofen et al., 1996). Increased NGF production within the ovary also results in disruption of estrus cyclicity (Dissen et al., 2000).

BDNF, NT-4, and NT-3 have all been detected in adult human follicular fluid (Seifer et al., 2003, 2002b). *In vitro* studies on human cumulus granulosa cells have also found that BDNF secretion can be stimulated by treatment with cAMP, forskolin (an activator of cAMP), human chorionic gonadotropin (hCG), menopausal urinary gonadotropin (a mixture of approximately equal FSH and LH bioactivities), but not recombinant FSH alone (Feng et al., 2003). Both BDNF and NT-4, but not NT-3 promote first polar body extrusion in cumulus-free mouse oocytes matured *in vitro*, compared with those matured in the absence of serum or hormones (Seifer et al., 2002a,b). Accordingly, by immunohistochemistry these studies have reported that most mouse oocytes are positive for TrkB, with no immunoreactivity for TrkC, the normal receptor for NT-3.

Recently, we have investigated the effects of BDNF on maturation of bovine oocytes, with the implications for embryo development assessed by parthenogenetic activation (Martins da Silva et al., 2004b). Parthenogenesis provides a means of assessing oocyte cytoplasmic competence to elicit development independently of sperm mediated factors, and is an accepted standard to assess oocyte viability for cloning and nuclear reprogramming. We have found that BDNF and the p75 receptor protein are expressed in preantral and antral follicles by granulosa cells and oocytes. Similarly, protein and mRNA for this ligand and receptor are expressed by cumulus cells and oocytes before and after maturation. In contrast to the mouse, mRNA for the full-length and truncated isoforms of the TrkB receptor are only present in cumulus, suggesting that oocytes and cumulus may differ in their capacity to respond to neurotrophin signalling. We have also confirmed a similar expression of BDNF ligand and receptors in both ovine (Fig. 1) and human cumulus-oocyte complexes (De Sousa et al., 2004, unpublished data). These similarities suggest that cow or sheep might provide better models with which to predict the consequences of neurotrophin signalling in human oocytes.

In bovine oocyte *in vitro* maturation experiments using hormone supplemented media (i.e., FSH, LH, and estradiol) as a base, supplementa-

tion with BDNF is inferior to supplementation with serum and equivalent to serum-free conditions, in the yield of cumulus enclosed oocytes which reach metaphase II by 27 h of maturation (i.e., a time by which they are ready to be artificially activated or fertilized). However, after maturation and activation, the proportion of oocytes which can form parthenogenetic blastocysts is improved with BDNF (relative to serum). This effect can be blocked by *in vitro* maturation in the presence of a BDNF blocking antibody (Martins da Silva et al., 2004b). Interestingly, BDNF alone with hormones does not support cumulus expansion to the extent observed with serum. Similar effects on MII yield and blastocyst formation are observed using oocytes matured without cumulus, indicating that BDNF may act directly on oocytes as well as cumulus. Parthenogenetic blastocyst cell numbers are unaffected by *in vitro* maturation culture supplemented with BDNF, serum, blocking antibody, or nothing. Thus, the effect of BDNF during *in vitro* maturation of oocytes appears to be in promoting oocyte competence to yield embryos, rather than the promotion of better embryos as such. Furthermore, it appears to do so without equally supporting nuclear maturation or cumulus expansion (Fig. 1).

Although we have yet to characterize whether other neurotrophin ligands (i.e., NGF, NT3 and 4) and receptors (Trk A and C) are expressed in bovine cumulus-oocyte complexes, our studies suggest that BDNF signaling between cumulus and oocytes may be bi-directional with different signaling pathways elicited in each cell owing to differentially expression of receptors (i.e., p75 and TrkB isoforms in cumulus, with the latter absent in oocytes). Trk and p75 receptors do not bind directly to each other, but can be complexed together (Bibel et al., 1999; Lee et al., 2001). This association results in higher affinity ligand binding and a greater discrimination between neurotrophins. Thus, BDNF, NT4, and even NT3 can bind to the TrkB receptor, but in the presence of p75 only BDNF provides a functional response (Bibel et al., 1999).

In neurons, Trk receptors and their substrates can activate three main signalling cascades: (1) differentiation, via a Ras/Raf/ MEK/MAP kinase pathway, (2) cell survival (anti-apoptosis), by association with insulin receptor substrates leading to inactivation of proapoptotic proteins, and (3) calcium release from internal stores via

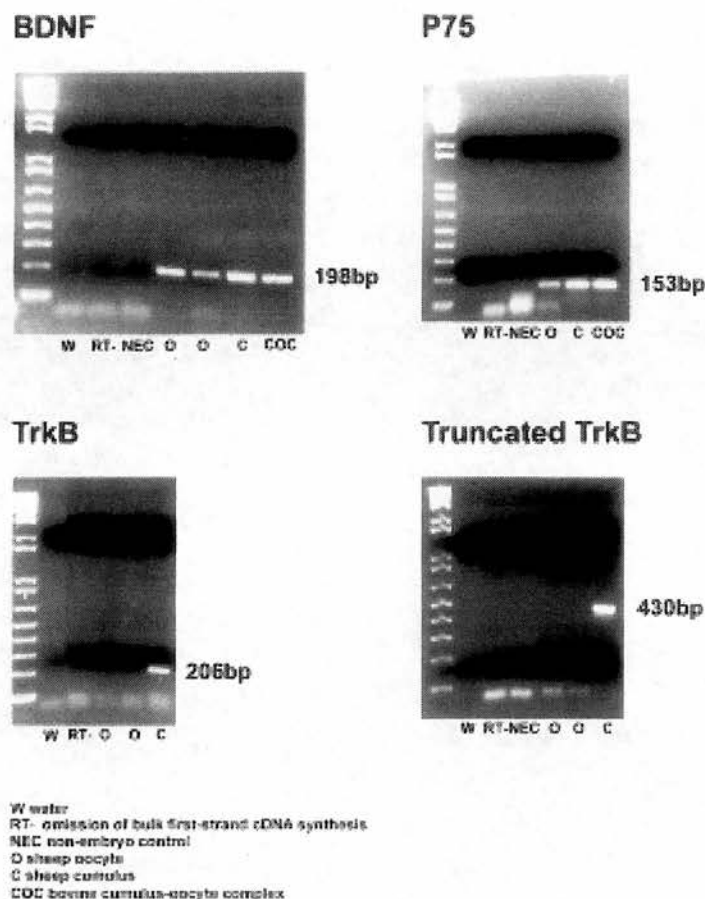


FIG. 1. Expression of BDNF, the p75 low-affinity neurotrophin receptor, and isoforms of the high-affinity Trk B receptor in ovine cumulus-oocyte complexes. Neurotrophin ligand and receptors were amplified by RT-PCR as described in da Silva et al. (submitted). Amplification products representative of BDNF (198 bp), p75 (153 bp), TrkB (206 bp) and truncated TrkB (Trunc TrkB, 430 bp) mRNA are detectable in RNA prepared from *in vitro* matured ovine cumulus oocyte complexes (COC) or isolated cumulus (C) cells. By contrast, only BDNF and p75 mRNA products can be detected in mature oocytes (O). As negative controls, PCR was performed on water (W), COC RNA that was not reverse transcribed (RT-) and a blank reverse transcription reaction lacking COC RNA (i.e., NEC, non-egg/embryo control).

PLC- γ mediated production of IP₃ and production of protein kinase C, which in neurons plays a role in neurotrophin mediated neurotrophin release (Bibel et al., 2000). All of these pathways may be relevant to follicular growth and differentiation and final oocyte ovulation and maturation. Gonadotropin induced cumulus expansion and resumption of meiosis in oocytes is dependent upon activation of MAP kinase in granulosa cells. This activation is downstream of gonadotropin-induced elevation of granulosa cell cAMP, and is dependent upon one or more paracrine factors from the oocyte (Su et al., 2003). BDNF secretion by cumulus is another consequence of either gonadotropin stimulation or artificial eleva-

tion of cAMP (Feng et al., 2003). Since oocytes and cumulus cells communicate via gap junctions and elevation of cAMP in cumulus leads to the same in oocytes, BDNF secretion by oocytes may also be a downstream consequence of gonadotropin stimulation. This could then act in an autocrine or paracrine fashion to augment the effect of cumulus-derived BDNF (Fig. 2). If in fact BDNF plays a role in cumulus expansion our data suggests that there is still a requirement for factor(s) provided by serum. It is unknown whether BDNF derived from oocytes or cumulus would differ in their bioactivity. Neurotrophins in general are substrates for pro-protein convertases that can alter the molecular bioactivity of their targets by

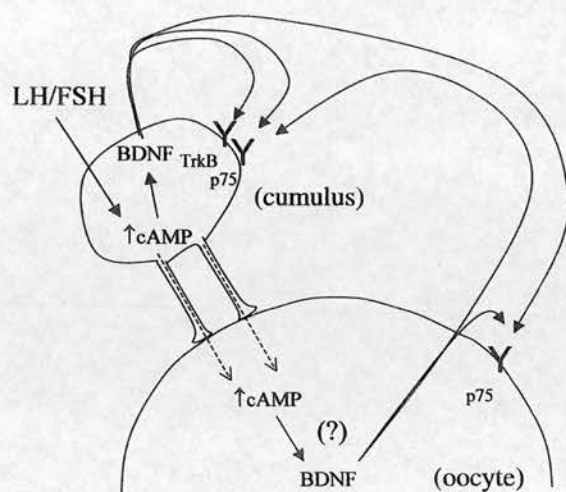


FIG. 2. Schematic of BDNF ligand and receptor expression in cumulus-oocyte complexes and potential signaling consequences. In bovine, ovine and human cumulus-oocyte complexes BDNF and p75 expression is observed in both cumulus and oocytes, whereas Trk B isoforms (full and truncated) are in cumulus cells only (Martins da Silva et al., 2004b; De Sousa et al., 2004, unpublished data). BDNF secretion by cumulus cells is downstream of elevated cAMP and stimulation by LH and FSH (Feng et al., 2003). Intercellular communication between cumulus cells and the oocyte via gap junctions is known to result in elevated oocyte cAMP. Theoretically, this could stimulate secretion of oocyte BDNF. As such, cumulus and oocyte derived BDNF could function in autocrine or paracrine signaling.

proteolytic cleavage during intracellular and extracellular processing. BDNF is an especially well characterized example of convertase-dependent modulation of bioactivity (Seidah et al., 1999). Thus, differences in BDNF bioactivity could be achieved by differential expression of pro-protein convertases in cumulus and oocyte. At present, however, there is no information about the expression of these molecules in this context.

As with Trk receptors, the functional consequences of ligand interaction with the p75 receptor are also complex, and in neurons have been linked to cell survival, arrest, differentiation, and programmed cell death. Recently, the concept of p75 as a "quality control" receptor has been advanced based on evidence that it is capable of mediating programmed cell death in response to either ligand binding or withdrawal. It suggests that the downstream consequence of ligand interaction is dependent on the relative proportions of p75 and Trk receptors and which neurotrophin is present (Rabizadeh et al., 2003). According to this model, p75 is proposed to suppress apopto-

sis (i.e., pro-cell survival) in the absence of Trk receptors. As a member of the tumor necrosis factor receptor superfamily, the stimulation of pro- or anti-apoptotic mechanisms via p75 is also dependent on the availability of different cytoplasmic factors (Chung et al., 2002). Recruitment of TNF-receptor associated factor 6 (i.e., TRAF6) to p75, leading to activation of NF- κ B, has been shown to result in cell survival in numerous cell types (Denchant, 2002). From mouse knockout models, mice homozygous for a targeted mutation in p75, rendering it functionally inactive, are viable and fertile, although they eventually develop deficits in their peripheral sensory nerves characterized by heat sensitivity and susceptibility to ulceration (Lee et al., 1994). However, since the expression of p75 in mouse fully grown antral follicles or oocytes has never been properly characterized, the significance of the null phenotype is unclear. Further experiments are therefore necessary to elucidate the role of p75 in both cumulus and oocytes. We hypothesize that expression in the latter in the absence of Trk receptors may promote oocyte survival, which would be especially critical to overcoming the stress of *in vitro* culture.

NEUROTROPHIN SIGNALING IN STEM CELL SURVIVAL

Neurotrophins are of course well established regulators of neuronal cell survival as well as differentiation (Bibel et al., 2000). *In vivo* this has been illustrated by targeted mutation of both ligands and receptors generally resulting in deficiencies in nervous system innervation or lesions (Ernfors et al., 1994; Lee et al., 1992; Schimang et al., 1995). *In vitro* this is evident in neural progenitor cells isolated from the cortical ventricular zone of the hippocampus of mouse fetal and neonatal brain. These cells secrete BDNF and NT3 and express their corresponding receptors, Trk B and C, as well as p75 (Barnabe-Heider et al., 2003; Hosomi et al., 2003). Inhibition of these neurotrophins using function-blocking antibodies results in a decreased survival of cortical progenitors, accompanied by decreased proliferation and inhibition of neurogenesis. This is achieved by the suppression of separate pathways mediating survival (i.e., phosphatidylinositol-3-kinase) and differentiation (MAP kinase) (Barnabe-Heider et al., 2003).

A role for neurotrophin signaling in the survival of other types of stem cells has yet to be demonstrated although some supporting evidence is provided by the detection of p75 and associated factors. Human esophageal keratinocyte stem cells are characterized by and can be isolated from differentiating derivatives by the expression of p75 together with the cell adhesion molecules, $\beta 1$ and $\beta 4$ integrins (Okumura et al., 2003). More significant is the outcome of a recent microarray comparison of differences between human and mouse embryonic stem (ES) cells. In contrast to the mouse, human ES cells highly express p75, and associated factors capable of mediating cell survival (i.e., anti-apoptosis), namely TRAF6, and BLK, the latter a Src family protein tyrosine kinase that participates in NF- κ B activation (Ginis et al., 2004). Recently, a functional interrelationship between the control of apoptosis and differentiation has been suggested in both mouse and embryo stem cells (Zwaka et al., 2004). In these cells, caspase activity mediating protein cleavage during cell death transiently increases upon induction of differentiation. Application of specific caspase blockers also causes differentiation. Since p75 can mediate both cell death and survival, signaling via this receptor may prove as relevant to stem cell survival, as it may be in the oocyte to preserve developmental competence.

CONCLUSION

In conclusion, oocyte developmental competence is gradually acquired over the course of oocyte growth and completed with maturation. It is dependent on bi-directional signaling with follicular granulosa cells. Neurotrophin signaling plays a key role in oocyte survival and follicular formation and growth initiated in fetal ovaries. In the adult ovary, this signaling is responsive to gonadotropins and may be involved in the control of ovulation. Our research adds neurotrophins, specifically BDNF, to the list of growth factors normally present in follicular fluid, which can specifically promote oocyte developmental competence during maturation. However, BDNF differs from most other growth factors, in that improved competence to support embryo development is not preceded by improved nuclear maturation or support of cumu-

lus expansion. Thus, BDNF plays a key role in the acquisition of oocyte cytoplasmic competence to support development. This effect is likely to be mediated by autocrine and paracrine signaling between cumulus cells and oocytes, which we hypothesize in the latter may stimulate cell survival mechanisms. This knowledge is of direct relevance to assisted reproductive technologies. However, as the original toti-potent cell, insight gained from an improved understanding of oocyte developmental competence may contribute generally to understanding the control of stem cell toti-potency and survival.

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